THE ROLE OF LIPID PEROXIDATION IN THE PATHOGENESIS OF DUCHENNE MUSCULAR DYSTROPHY

Jamaludin bin Mohamed

A Thesis Submitted for the Degree of PhD
at the
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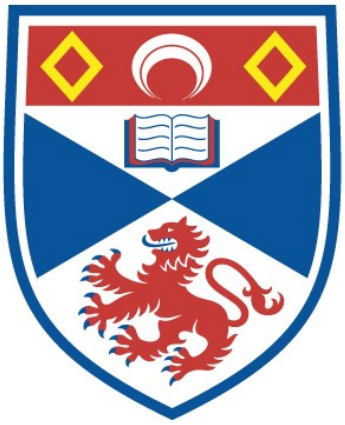

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by

JAMALUDIN BIN MOHAMED
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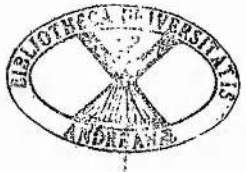

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DECLARATION

I hereby declare that this thesis has been composed by myself, and that it is a record of work which has been done by myself. None of the work has been accepted in any previous application for a degree. Any other sources of information have been acknowledged.

Signed

(Jamaludin bin Mohamed)

Statement

I, Jamaludin bin Mohamed, was admitted as a research student of the University of St. Andrews in October 1981 in accordance with Ordinance General No. 12 and the Resolution of the University Court, 1967, No. 1. The thesis was completed in October, 1984.

Certificate

I hereby declare that Jamaludin b. Mohamed has been engaged upon research from October 1981 onwards to prepare the accompanying thesis for the degree of Doctor of Philosophy.

Signed

(Dr M. I. S. Hunter)

St. Andrews

October 1984

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Dedicated to my father (Mohamed Nong), my wife (Norziah Mahmud) and my son (Muhd. Khairul Nizam) for their patience, sacrifice, understanding, and constant encouragement during my endeavor for the completion of this important goal in my life.

ABSTRACT

Duchenne muscular dystrophy (DMD) is a progressive muscle disease which is eventually fatal due usually to respiratory and cardiac failure. Although DMD is known to be an inherited, X-linked, disease the nature of the genetic defect remains elusive. Biochemical and histological evidence points to a lesion in the cell membranes of muscle and possibly other cell types although the nature of this lesion has not been elucidated. One of the many theories is that membrane lipid peroxidation causes the membrane damage and consequent necrosis seen in DMD. The aim of this work was to search for evidence relating to this theory using cultured skin fibroblasts (CSFs) and blood plasma.

It was demonstrated that whole cultures of DMD CSFs, show markedly increased MDA production which distinguished them from normal control CSFs. Both CSFs grown in a medium without PUFA and with PUFA showed that lipid peroxidation occurs in a time-dependent manner and higher levels of MDA were found in control and DMD CSFs incubated in PUFA. It was also found that washed CSFs showed a considerable increase in MDA production compared with unwashed ones. The concentration of tert-butyl hydroperoxide (TBH) which is just below the minimum toxic level for CSFs was determined and found to be 50μmol/l.

Although lipid peroxidation products (MDA and FP) were not significantly different in the washed particulate fraction from DMD and normal control CSFs, these products (CD, MDA and FP) were significantly higher in total homogenates from DMD compared with

normal control CSFs suggesting that membrane lipid peroxidation may not be entirely membrane-dependent but also probably involves cytoplasmic components. Protection by a GSH-dependent cytosolic factor from CSFs against lipid peroxidation in a model system was examined. This work strongly indicates that DMD CSFs have a better protection system than normal controls.

All three lipid peroxidation products (CD, MDA and FP) were measured in plasma and found to be significantly increased in DMD ($P<0.02$; $P<0.01$; $P<0.001$ respectively) compared with normal control. The stability of the products (CD, MDA and FP) in DMD and normal control plasma was examined during storage for up to 3 years at $-20^{\circ}C$. There was a significant increase of MDA and FP concentrations with time but no significant changes in CD for both DMD and controls with time of storage. There was a significant positive correlation between MDA concentration and age but not for CD and FP concentrations.

Total plasma antioxidant activity (AOA) is markedly higher in DMD (76%) than in normal controls (63%) and significantly different ($P<0.001$). AOA and MDA were positively significantly correlated ($P<0.05$) in DMD plasma. Plasma vitamin E concentration is significantly lower ($P<0.002$) in DMD than in normal controls (4.14 ± 2.00 compared to 9.30 ± 2.45 mg/litre) but not significantly correlated with plasma AOA ($P>0.05$). A general but small decrease in alpha-tocopherol was seen in both DMD and control during storage ($-20^{\circ}C$) up to 3 years. There was no correlation between age and vitamin E concentration for a group of adults (ages 18-35) and children (ages 3-14).

Lastly plasma transferrin concentration shows no significant

difference between DMD and normal controls ($P>0.05$) but a significant increase in caeruloplasmin was found in DMD patients compared with normal controls ($P<0.001$). Neither levels of transferrin nor caeruloplasmin (in DMD and normal controls) were influenced by time of storage ($-20^{\circ}C$). Further both proteins showed no significant difference with age of subject.

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ABBREVIATIONS

ADP	Adenosine diphosphate
AOA	Antioxidant activity
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BHT	Butylated hydroxy toluene
CAT	Catalase
CD	Conjugated diene
CK	Creatine kinase
CSFs	Cultured skin fibroblasts
CuZnSOD	Copper and Zinc-containing superoxide dismutase
DMD	Duchenne muscular dystrophy
DMSO	Dimethylsulphoxide
DNA	Desoxyribose nucleic acid
E	Extinction coefficient
E face	External face
EDTA	Ethylenediaminetetra-acetate
Em	Fluorescence emission
EM	Electron microscopy
ESR	Electron spin resonance
Ex	Fluorescence excitation
FBS	Foetal bovine serum
FeSOD	Iron-containing superoxide dismutase
FP	Fluorescent pigments
G6PDhse	Glucose-6-phosphate dehydrogenase
GLC	Gas-liquid chromatography
GSH	Glutathione
GSHPx	Glutathione peroxidase
GSHR	Glutathione reductase
HCl	Hydrochloride acid
HDL	High density lipoprotein
HPLC	High-performance liquid chromatography
I(1.14)	2-(14-carboxyltetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl
I(5.10)	2-(10-carboxyldecyl)-2-hexy-4,4-dimethyl-3-oxazolidinyloxyl
I(12.3)	2-(3-carboxylpropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl
LDL	Low density lipoprotein
LO°	Lipid alkoxyl radical
LOOH	Lipid hydroperoxide
lysoPC	lysophosphatidylcholine
MAL-6	Maleimide nitroxide
MDA	Malondialdehyde/Malonyldialdehyde
MnSOD	Manganese-containing superoxide dismutase
MyD	Myotonic muscular dystrophy
MW	Molecular weight
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NBS	Newborn bovine serum
NMR	Nuclear magnetic resonance
5-NMS	2-(3-carboxylpropyl)-2-tridecyl-4,4-dimethyl-3-oxazolidinylloxyl methyl ester (5-nitroxide methyl strearate)
16-NMS	16-nitroxide methyl strearate
NS	Not significant
5-NS	5-nitroxide strearate
$^1O_2$	Singlet oxygen
OD	Optical density
$^{\circ}OH$	Hydroxyl radical

P face	Protoplasmic face
PC	Phosphatidyl choline (Lecithin)
PE	Phosphatidyl ethanolamine
PGE_1	Prostaglandin E_1
PGE_2^1	Prostaglandin E_2^1
PI	Phosphatidyl inositol
PNPPase	p-nitrophenylphosphatase
PS	Phosphatidyl serine (cephalins)
PUFA	Polyunsaturated fatty acids
$PUFA^{\circ}$	PUFA free radical
$PUFAOO^{\circ}$	PUFA peroxyl radical
PUFAOOH	PUFA hydroperoxide
RBC	Red blood cells
RNA	Ribonucleic acid
RO°	alkoxyl radical
ROO°	peroxyl radical
ROOH	Hydroperoxide
S.D	Standard deviation
SDS	Sodium dodecyl sulphate
SM	Sphingomyelin
SOD	Superoxide dismutase
SR	Sarcoplasmic reticulum
SV	Scintilation vial
TBA	Thiobarbituric acid
TBH	tert-butyl hydroperoxide
TCA	Trichloroacetic acid
TG	Triglyceride
TLC	Thin layer chromatography
U.V	Ultraviolet
VIS	Visible
VLDL	Very low density lipoprotein
16:0	Palmitic acid (n-hexadecanoic)
16:1	Palmitoleic acid (cis-9-hexadecenoic)
18:0	Stearic acid (n-octadecenoic)
18:1	Oleic acid (cis-9-octadecenoic)
18:2	Linoleic acid (cis-9,12-octadecadienoic)
18:3	Linolenic acid (cis-9,12,15-octadecatrienoic)
20:4	Arachidonic acid (5,8,11,14-eicosatetraenoic acid)
20:6	cis-4,7,10,13,16,19-docosahexaeinoic

1 INTRODUCTION

1.1 MUSCULAR DYSTROPHIES

Any muscle diseases in which there are some pathological, biochemical or functional lesion in muscle fibre or in the interstitial tissue of voluntary musculature are generally recognized as "myopathies". Inherited diseases of skeletal muscle are one of the group of diseases which are classified as the myopathies. In 1850 Aran published the earliest report of cases of progressive muscular wasting and weakness. Five years later, Wachsmuth (1855) described a large number cases of primary muscle diseases. Then, in 1868, Duchenne published a classical description of pseudohypertrophic muscular paralysis in children. Walton and Gardner-Medwin (1981) classified all those myopathies which are in nature primary, genetically determined, progressive and degenerative as "muscular dystrophies". All seriously curtail normal activities, and prevent enjoyment of an active life. Eventually, they cause death by interfering with vital functions such as breathing, coughing and swallowing as well as affecting the heart. Structural or chemical faults in the machinery of the muscle cells disturb muscle functions. Disturbances in the chemical environment or failure of proper control by its nerve may result in weakening of the muscle. The most serious, commonest and most investigated type of muscular dystrophy is Duchenne muscular dystrophy (DMD).

1.1.1 DUCHENNE MUSCULAR DYSTROPHY

1.1.1.1 GENETIC STATUS

DMD is an inherited disease of the X-linked recessive type. Only males are therefore normally affected. Estimates of its incidence range from 13-33 per 100,000 live male birth. It is a particularly severe form of muscular dystrophy and it has also been claimed to have one of the highest spontaneous mutation rates. These claims arise from several studies (Roses et al.,1976; Zellweger et al.,1980) which link the high mutation rate with the high proportion of "sporadic" cases of the disease : the birth of boys with DMD in families with no previous history of it and to the high proportion of mothers of boys with the disease who were negative in various biochemical tests for the carrier state. Almost all males with the disease die without reproducing but the incidence of the disease appears not to be falling. Starting from this basis, Roses et al (1976) proposed that a high mutation rate replaces the genes lost in each generation. Carriers have an XX-genotype with one X-chromosome carrying the DMD gene abnormality which, because it is recessive, is masked by normal factors on the other X-chromosome. Thus, the disease is transmitted through females who are usually asymptomatic. There is a 50% probability that the male offspring of a carrier will be afflicted, and a 50% probability that the female offspring will be a carrier (Zundell and Tyler, 1965). However, there are cases where females show full symptoms of DMD. In the case of the XO-genotype the disease is fully manifested by female carriers. An example of this condition is in Turner's Syndrome. Turner's Syndrome girls with DMD have been found (Walton, 1956; Ferrier et al, 1965). Full manifestation would, of course, also occur in homozygotes, where the expression of the abnormal gene cannot be masked by the presence of

the normal allele on the second X-chromosome.

1.1.1.2 CLINICAL FEATURES AND DIAGNOSTIC SIGNS

1.1.1.2.1 CLINICAL FEATURES

The clinical onset of the disease is in early childhood (2-3 years old) but it is now generally believed that the real biochemical lesions appear from birth, and possibly much earlier. The initial symptoms of the disease are difficulty in climbing stairs and rising from the floor, due to weakness which develops first in the proximal musculature of the pelvic girdle and then spreads to the shoulder girdle. The disease has a rapid progression during which the wasted muscle tissue is replaced by fat and connective tissue (pseudohypertrophy). Eventually this involves all the voluntary muscles along with skeletal changes. The unfortunate individual is usually confined to wheel-chair by the age of 11 as the relentless loss of muscle function continues. Death is usually due to cardiac failure or respiratory infection in the late teens or early twenties. Frequently patients are mentally retarded and there is cardiac involvement.

1.1.1.2.2 PLASMA ABNORMALITIES

The monitoring and diagnosis of both carriers and patients with DMD currently relies on measurement of the activity of certain muscle-specific enzymes in the plasma. Creatine kinase (CK) is the enzyme most widely used for this purpose since it is thought to be the most sensitive index of muscle breakdown, although myoglobin, pyruvate kinase, hemopexin, and other glycolytic enzymes, have also been used (Pennington, 1980). It is generally assumed that the elevated levels of enzymes in DMD are due to leakage through a defective muscle membrane (Rowland, 1980).

1.1.1.2.3 PRENATAL DIAGNOSIS

Cultured amniotic cells are sometimes important in the prenatal diagnosis of serious X-linked diseases providing there is expression of the defective gene in these cells. However, because at present there are no clear-cut, easily measurable abnormalities in these cell types in DMD this technique is not used. Nevertheless, the possibility of prenatal diagnosis of DMD has improved with the introduction of foetoscopy into medicine (Benzie et al.,1980). This technique has already been found useful in diagnosis of inherited anomalies and fetopathologies (Gustavii et al.,1983). Thus, foetal blood sampling from the placenta has became possible, although results to date for CK from DMD foetuses are equivocal.

1.1.1.3 THEORIES FOR THE PATHOGENESIS OF DMD

Several theories have been proposed to explain the pathogenesis of DMD. These include the Vascular and Neurogenic Theories. Neither of these has received experimental support, although both stimulated research that clarified many "potential" points. Alternatively, the popular theory currently is that DMD may result from some abnormality of the sarcolemma (Membrane Theory). These theories are discussed separately in the following sections.

1.1.1.3.1 THE VASCULAR THEORY

As the name implies, the Vascular theory in all its forms proposes an abnormality of muscle vasculature (usually the microcirculation) resulting in an insufficient or inefficient blood supply. Muscle injury is usually attributed to ischemia. That inadequate blood flow might account for the degeneration of dystropic muscle was proposed as long ago as 1930 by Kure and Okinada. The idea

has been proposed repeatedly over the past century by several workers including Duchenne himself but apparently was not taken too seriously until Demos (1961) found slowed arm-to-tongue circulation times in patients and carriers. A later study suggested an abnormality of catecholamine metabolism due to deficient oxidation of these compounds in platelets (Demos, 1973) as well as abnormal serotonin uptake in DMD platelets (Murphy et al, 1973). Accumulation of unidentified catecholamines was observed in dystrophic muscle by fluorescence microscopy (Wright et al,1973). The patterns of O_2 transport and consumption were expected to alter due to changes in the composition and architecture of muscle. A related study found significantly decreased intramuscular O_2 tension in DMD (Kunze and Olthoff, 1970), but again it is unclear how this might be related to the various aspects of O_2 transport and consumption.

1.1.1.3.2 THE NEUROGENIC THEORY

The spinal cord and anterior horn cells are suggested to be involved in the Neurogenic theory. McComas et al (1971) proposed that the muscular dystrophies result from "sick motor neurones". But this theory has been chellenged (Montgomery, 1975). Ballantyne and Hansen (1974), using a new method for the estimation of the number of motor units in muscle, from DMD, limb-girdle, fascioscapilohumeral myotonic muscular dystrophies found no loss of motor neurones. Motor-end-plates and nerve terminals were studied using morphometric analysis (Wirtz et al.,1983) but again no abnormality was found. In conclusion, the present status of the neurogenic theory in pathogenesis of DMD is uncertain and is likely to remain so until more conclusive experimental evidence is forthcoming.

1.1.1.3.3 MEMBRANE THEORY

It is now generally accepted that there is little satisfactory evidence for the previous two hypotheses. The high serum enzyme levels originating from muscle tissues, and the general imbalance of metabolites in cases of myopathy, suggest that there might be a genetically determined structural defect in the muscle cell membranes expressed as a "leak". A great deal of evidence to support the membrane theory has been published on muscle fibers/cells as well as non-muscle cells such as red blood cells (RBC), lymphocytes, adipose tissue and cultured skin fibroblasts (CSFs) which suggest a generalised membrane defect in DMD. This evidence has been the subject of two comprehensive reviews (Rowland, 1980; Lucy, 1980). A brief review of those studies which support the generalised membrane theory will be discussed in the following sections. In addition, the Ca^{2+} theory as an extension of the membrane theory is currently the most popular one: several investigators believe that an abnormal influx of Ca^{2+} is another manifestation of abnormal permeability or physical interruption of the cell surface membrane in DMD (Publicover et al.,1978). However, this notion seems to have been suggested first by Cullen and Fulthorpe (1975) in their attempt to explain why "hypercontracted" or "opaque" fibres are seen so frequently in DMD biopsies (Boxler and Jerusalem, 1978). They suggested that areas of abnormal permeability might allow influx of Ca^{2+} in amounts sufficient to cause excessive contraction of some sarcomeres. This in turn would excessively stretch adjacent sarcomeres and some of the stretched myofilaments would be disrupted, initiating necrosis. They suggested that this might be repaired at first, but sooner or later the reparative and regenerative processes would fail to keep pace, and the muscle would degenerate. Wrogemann and Pena (1976) proposed another

kind of Ca^{2+}-related theory of muscle cell necrosis. They also assumed that the influx of Ca^{2+} is excessive in dystrophic fibres. The resulting increased intracellular Ca^{2+} content would stimulate Ca^{2+} uptake by mitochondria. If this uncontrolled uptake/influx of Ca^{2+} occurs, mitochondria will sequester increased amounts of Ca^{2+}, thereby diverting the electron transport chain from ATP formation to Ca^{2+} transport. As a result, less energy is available for the ATP-dependent, Ca^{2+}-pumping mechanism in the SR and plasma membrane, as well as for another essential cell functions, perhaps including those responsible for maintaining the integrity of the cell surface membranes. Finally, others have pointed out that an abnormal influx Ca^{2+} might stimulate Ca^{2+}-activated proteases to foster the process of myofibrillar degeneration (Pennington, 1977) and that the surface membrane itself is involved in the regulation of intracellular Ca^{2+} content (Sulakhe and St.Louis, 1976).

1.1.1.3.3.1 MUSCLE ABNORMALITIES

1.1.1.3.3.1.1 HISTOLOGICAL CHANGES

1.1.1.3.3.1.1.1 LIGHT MICROSCOPIC STUDIES

Light microscopy can be used to observe most pathological changes associated with DMD (Adams, 1981). Among them are necrosis, phagocytosis, fibre enlargement, branching of the fibers, enlargement of sarcolemmal nuclei, fat cell infiltration and increased connective tissue mass. The stage of disease at which the biopsy, for light microscopic examination, is taken is important. Each stage gives different observations. Two stages of the disease were studied by Hudgson (1971): (i) fibers from advanced cases of DMD and (ii) fibers which had recently undergone necrosis. These stages are ultrastructurally different from one another. An amorphous mass of fibers and a few mitochondria within the mass of fused filaments were observed in the first type. Changes were more easily recognizable,

showing widespread dilation of SR, a grey granular sarcoplasm and some regenerative activity, in necrotic fibres. These studies were continued by Engel (1977) who suggested that the histopathological changes in DMD were characterized by the features of other myopathies. In addition, a few groups have used the dye, procion yellow as an extracellular marker to look for muscle cells with damaged membranes (Bradley and Fulthorpe, 1978). The permeation of procion yellow was observed at the light microscopic level in dystrophic muscles biopsies, but not in controls.

1.1.1.3.3.1.1.2 ELECTRON MICROSCOPIC STUDIES

An attempt to correlate the alterations in serum CK activity with the primary lesions which initiated fibre breakdown in DMD, was made using electron microscopy (EM) (Milhorat et al.,1966). It was concluded that fibre degeneration is divided into three sequential phases based on the condition of fibres. Cullen and Fulthorpe (1975) later proposed five stages involved in fibre breakdown: (i) fibers appear normal, but less mitochondria and more SR than normal fibres, (ii) fibres were overstretched, (iii) localized contraction has continued to the extent that the stretched area between the clumped myofibrils are partly empty of contractile material, (iv) the clumps of contractile material is further condensed and it outlines is more rounded, (v) the fibres, which are progressively invaded by marcophages, now consist only of structureless cytoplasm containing no contractile material. Occasionally regenerating fibres are found in the vicinity of these final remnants. In another study focal defects in the plasma membrane from patients with DMD were described (Mokri and Engel, 1975), where the plasma membrane was disrupted or absent, however, the basement membrane was always preserved. It was suggested that the alterations in the plasma membrane were possibly an early and

basic lesion in DMD. Later this observation was chellenged by suggestions that these findings could have been arterfacts due to the method of sample preparation (Rowland, 1980). Nevertheless, the fact that these lesions, even if artifactual, are only seen in dystrophic muscle, suggests some fundamental membrane instability.

1.1.1.3.3.1.2 SARCOPLASMIC RETICULUM FUNCTION

Muscle contraction and relaxation is regulated by membranes, of which the SR serves a central role. Thus, one of the functions associated with this membrane structure is the uptake and release of Ca^{2+} ions. Initial work on the SR in dystrophic muscle was carried out by Sreter et al (1964) on a preparation from dystrophic mouse and chicken muscle. They showed that dystrophic SR preparations had a reduced Ca^{2+} uptake as well as an increase in Mg^{2+}-activated ATPase activity. However, the first attempt to study DMD SR was by Sugita and his colleagues (1967) who showed that Ca^{2+} uptake was reduced. The Ca^{2+} uptake process was studied in more detail by Samaha and Gergely (1969). Unfortunately, the EDTA-sensitive portion of the ATPase activity was not studied. However, their data did allow measurement of the efficiency of the Ca^{2+}-uptake mechanism. In normals, the ratio of moles of Ca^{2+} taken up to moles of ATP hydrolyzed was 0.43 and in DMD muscle it was 0.56, clearly not lower than normal. Peter and Warsfold (1969) used a different approach to study Ca^{2+} uptake characteristics of DMD SR. They also found a reduced rate of uptake and total uptake of Ca^{2+}, but additionally studied so-called Ca^{2+} affinity, attempting to approach physiological conditions more closely by testing the ability of SR to remove Ca^{2+} from more dilute solutions. On incubation with 20μM Ca^{2+}, normal SR vesicles quickly reduced the Ca^{2+} concentration of the medium to less than 0.3μM, which is in the range required to induce relaxation of

muscle or superprecipitation of actomyosin. None of 5 cases of DMD showed a normal Ca^{2+} affinity of the SR. Fifteen other cases of a variety of neuromuscular diseases showed no abnormality of Ca^{2+} affinity. A similar abnormality, however, was found in polymyositis and in one patient with BMD. Peter and Warsfold (1969) considered Ca^{2+} affinity more sensitive because in DMD muscle, SR might show normal Ca^{2+} accumulation, but abnormal Ca^{2+} affinity. In 3 of the cases with abnormal Ca^{2+} affinity, isolated mitochondria showed normal respiratory rates, acceptor ratios, respiratory control ratios and ADP/O_2 ratios. They concluded that the SR is abnormal in DMD at a time when mitochondrial function appears to be normal. Takagi and his colleagues (1973) also reported that the initial rate of uptake and the total uptake of Ca^{2+} by DMD SR were slower and lower than normal. However, the Mg^{2+}-ATPase of the SR was little changed. The work regarding Ca^{2+} and DMD SR has been reviewed by Samaha (1979). Membrane-related ATPase ($[Na^{+}+K^{+}]$ATPase; $[Ca^{2+}+Mg^{2+}]$ATPase; and $[Na^{+}+K^{+}]Mg^{2+}$-dependent ATPase) activity and the content of serine phosphatides were decreased in DMD SR (Peter et al.,1974). Recently, Niebroj-Dobosz (1981) reported that the diminution of membrane-related ATPase activity in their patients with DMD and MyD, was not restricted to any one of these dystrophies, suggesting that it was not the cause but the consequence of the genetic abnormality of the muscle surface membrane. Decreased $[Na^{+}+K^{+}]$ATPase and $[Ca^{2+}+Mg^{2+}]$ATPase activity could result from either altered activator/inhibitor actions or disorganization of the molecular structure of the membrane (Charnock et al., 1971).

1.1.1.3.3.1.3 FREEZE-FRACTURE IN DYSTROPHIC MUSCLE

Freeze fracture is a unique technique. It allows the electron microscopist to visualize and quantitate fine details of membrane

ultrastructure. Branton and his colleagues demonstrated that the fracture consistently passes along the midline of the membrane; that is, through the hydrophobic phospholipid interior. Both fracture faces are covered with particles and it is now accepted that these particles are proteins and structural components of the membrane Marchesi et al.,1972). Since previous biochemical and morphological studies implicated a muscle surface membrane abnormality in DMD (Mokri and Engel, 1975), the intramembranous architecture of the plasma membrane in DMD muscle has been studied (Schotland et al.,1981). These reports agreed that there are decreased numbers of intramembranous particles as well as changes in the distribution in the plasma membrane in the population of muscle fibres from patients with DMD. In conclusion, their studies support the view that a membrane abnormality is present in this disease although the identity of the particles is unclear.

1.1.1.3.3.2 ABNORMALITIES IN OTHER CELL TYPES

1.1.1.3.3.2.1 RED BLOOD CELLS (RBC)

RBC have received particular attention since the publication in 1967 of the first reports on abnormalities in RBCs from patients with muscular dystrophy (Brown et al.,1967). The abnormalities which have been found so far can be largely grouped into 3 categories: (i) morphological and physical membrane properties (ii) membrane-bound enzyme activity (iii) transport properties. Many of these abnormalities are contraversal and have been the subject of contradictory reports from different laboratories (Rowland, 1980).

1.1.1.3.3.2.1.1 RED CELL MEMBRANE PROTEINS

1.1.1.3.3.2.1.1.1 SPECTRIN

Spectrin is located on the cytoplasmic surface of the membrane

and is a major component of the RBC membrane. Along with other proteins such as actin it forms the cytoskeletal network responsible for the shape, strength, deformability and elasticity of the RBCs (Singer, 1976; Painter et al.,1975; Wang et al.,1975). Guidotti (1972) referred to the hypotonic EDTA-extracted proteins of the RBC membranes as 'RBC actomyosin'. These extracts consist mainly of band 1 and band 2 (spectrin) and band 5 (actin). Band 5 has many properties similar to muscle actin, including molecular weight, capacity to stimulate myosin ATPase activity, and ability to 'decorate' its fibrous form with muscle heavy actomyosin (Painter et al., 1975). Regarding spectrin from DMD RBCs, Roses et al (1980) found that band 2 (M.W 220,000)(Fairbanks et al.,1971) was subject to increased phosphorylation in DMD. It is unlikely that a change in protein kinase activity is solely responsible for this increase since none of the other endogenous membrane proteins are affected. This suggests that the substrate (spectrin band 2) itself might be altered in some way. Indeed, Roses et al (1980) has isolated specific regions of band 2, by controlled enzymic proteolysis and HPLC, in which resides the abnormality of phosphorylation. Previously, Roses (1977) suggested structural similarities between human spectrin and human muscle myosin. He speculated that since the muscle fibre degenerates in DMD, this means that myosin of muscle fibre is defective. He concluded that the same gene was involved in specifying spectrin and possibly one form of the heavy chain of muscle myosin (Roses et al.,1976).

1.1.1.3.3.2.1.1.2 FREEZE-FRACTURE STUDIES

Using freeze-fracture electron microscopy (Wakayama et al (1979) found a decrease in the number, and a change to non-uniform distribution, of intramembranous particles [P face (protoplasmic) and

E face (external)] in DMD RBC membranes. They suggested this was the consequence of a change in properties of band 3 polypeptides or in the membrane lipid with which they interact to form the intramembranous perticles. Further quantitative freeze-fracture electron microscopy, reviewed by Shotton (1982), has confirmed these findings.

1.1.1.3.3.2.1.2 PHYSICAL PROPERTIES

1.1.1.3.3.2.1.2.1 SHAPE AND DEFORMABILITY

The techniques of microsieving and micropipetting have been used in these investigations to examine RBC physical properties. One of the physical properties of DMD RBC that has been described as abnormal is that of deformability as determined by micropipetting which is decreased (Tillman et al.,1979). However, more recent measurements of pressure drop-velocity relations (microsieving technique) were carried out in a capillary pore rheometer using pure RBC suspensions. The study showed that DMD RBC deformability is not different from normal (Conlon et al.,1983), results which are in substantial agreement with similar microsieving studies by Nash and Wyard (1982).

In addition, studies of RBC shape by scanning electron microscopy and light microscopy have also suggested a membrane abnormality in DMD. Some groups (Grassi et al.,1978) have shown an increased proportion of echinocytes (spiculated crenated cells) in DMD RBC and other types of dystrophies. Others have found no increase in echinocytes but an increased proportion of stomatocytes (cup shaped RBCs) as distinct from the normal biconcave cell (Miller et al.,1976). These findings were also observed in carrier females. However, the abnormal shape observed under the electron microscopy could have been caused during preparative techniques subsequent to fixation as well as effect of long period storage. In addition, some

workers were found normal RBC deformability (Tillman et al.,1979).

1.1.1.3.3.2.1.2.2 OSMOTIC FRAGILITY

The report that RBCs from patients with DMD have irregular, crenated surface membranes seen by scanning electron microscopy (Matheson and Howland, 1974) prompted Fisher and associates (1976) to investigate osmotic fragility. They found increased osmotic fragility of RBCs in approximately 85% of the patients. More recently, Somer and his colleagues (1979) also found increased osmotic fragility of RBCs in about 50% of DMD patients, but Adornato et al (1977) reported normal osmotic fragility and RBC turnover rate from DMD patients (i.e. the survival of DMD RBCs is normal). Three other publications reported increased osmotic fragility (Lloyd and Nunn, 1978). Interactions between membrane proteins and phospholipids can be modified by treatment of RBCs with phospholipases. These enzymes might affect DMD RBCs differently from control RBCs, if the mozaic of membrane components is altered. It is likely that this might then be reflected by a change in osmotic resistance. Ruitenbeek et al (1979) disproved this speculation by finding that after phospholipase A_2 (EC 2.1.1.4) treatment (enzyme from pancreas, snake venom or bee venom) there was no difference between the osmotic fragility of DMD and control normal RBCs, both being increased by the same amount.

1.1.1.3.3.2.1.2.3 SPECTROSCOPIC STUDIES

Electron Spin Resonance (ESR), Fluorescence and Nuclear Magnetic Resonance spectroscopy (NMR) have been well established as valuable tools in the study of both biological membranes and model membranes, since their introduction by McConnell and his co-workers in the middle 1960's. Spin labelling methods have been recently reviewed by Berliner (1976). A multitude of publications show that

spin-labelling has been applied with success to obtain detailed information on molecular organization and especially to measure the fluidity at different depths of the lipid bilayer of membranes. Many contradictory publications have appeared concerning lipid fluidity of the polar (surface) region, hydrophobic (non-polar) core and the protein conformation of DMD RBCs. The interesting studies by Butterfield et al (1976) as assessed using 5-nitroxide methyl stearate (5-NMS) implying differences in the spin label signals of RBCs from normal subjects and patients with myotonic muscular dystrophy (MyD) prompted Sato et al (1978) to use ESR [as assessed using I (1.14), I (12.3), and I (5.10)] to study RBCs from DMD. They found that the fluidity of the membrane near the polar region of DMD RBCs was similar to that of control normal RBCs. Their finding has been confirmed by Butterfield et al (1980) (as assessed using MAL-6 and 5-NMS) and Laurent et al (1980) (as assessed using 5-NMS and I(1.14)). Using saturation transfer ESR to study 5-NS labelled RBC from DMD and controls RBC, however, Wilkerson et al (1978) found decreased lipid fluidity at the surface (polar) region of DMD ghosts compared with control RBC ghosts. Later, Dunn (1980) confirmed their finding using the same probe (5-NS). On the hand, Dellantonio et al (1980), using the same probe (5-NS) , found increased lipid fluidity as well as alterations in lipid-protein organization. Recently, Falcioni (1982), reported the membrane environment in the polar region was different from that of normal RBC, as judged by lipid-soluble spin labels. They also found depletion of extrinsic proteins and intramembranous particles. Regarding lipid fluidity in the hydrophobic region of DMD RBC membranes, Sato et al (1978) reported it to be increased and this was also supported by Falcioni et al (1982). However, two groups, found there was no difference from that of normal RBC, judged by the spectra with C-12 and 16-NMS as probes, respectively. Furthermore,

protein conformation of DMD was also studied using ESR. As before, contradictory results were also found. Dellantonio et al (1980), using MAL-6 as probe found the architecture of the membrane proteins of DMD was altered compared to that of normal RBC. However, Laurent et al (1980) reported normal protein conformation of DMD RBC membranes. Maleimide nitroxide III and MAL-6 were used for their studies, respectively. In addition, Sarpel et al (1981) measured the lipid profile (especially PC) in whole RBC of DMD patients by ^{31}P NMR, and found a normal lipid feature. Clearly from this review, results from different investigators are contradictory. The reason why the results are not consistent is not clear, but one possibility may be that not all used the same probe for investigation of a given region (polar or unpolar) of the bilayer. Using different modes of ESR also undoubtedly affects the results.

1.1.1.3.3.2.1.3 ION TRANSPORT AND ATPases

Active ion transport in RBC membranes is regulated by membrane-bound ATPases. Human RBC membranes appear to contain 3 major ATPases, one which is activated by Mg^{2+} alone (Mg^{2+}-dependent ATPase), one (inhibited by ouabain) which requires Na^+,K^+,Mg^{2+}, ([Na^++K^+]ATPase or [Na^++K^+]Mg^{2+}-dependent ATPase), and a third one requiring Mg^{2+} and Ca^{2+} ([Ca^{2+}+Mg^{2+}]ATPase) (Siddiqui and Pennington,1977). Brown et al (1967) first reported that the membrane-bound ATPase, [Na^++K^+]ATPase, is enhanced by ouabain, a glycoside. Brown's work prompted many other groups to study the effect of ouabain on [Na^++K^+] ATPase. Their results can be summarised as follows: (i) enzyme activity was enhanced by ouabain (Pearson, 1978). (ii) enzyme activity was inhibited by ouabain less than controls (Siddiqui and Pennington, 1977) (iii) Basal enzyme activity was lower than control (Souweine et al.,1978). (iv) enzyme activity of DMD RBC was as in normal (Hodson and Pleasure,

1977).

[Ca^{2+}+Mg^{2+}] ATPase [EC 3.6.1.3] is the enzyme responsible for active Ca^{2+}-extrusion from RBCs (Ruitenbeek, 1979). Several groups have reported an increased [Ca^{2+}+Mg^{2+}] ATPase activity in DMD RBC (Pijst and Scholte, 1983). Thus led some authors to conclude that DMD might be caused by altered Ca^{2+} transport across the membrane leading to higher internal Ca^{2+} concentration in the cell. However, measurement of Ca^{2+} flux of DMD RBC (Shoji, 1981) did not show a significant difference between DMD and control preparations. An enzyme with some characteristics identical to those of Ca^{2+}-ATPase is the Ca^{2+}-stimulated p-nitrophenylphosphatase [Ca^{2+}+PNPPase]. It is also membrane-bound and it has been suggested (Rega et al.,1973) that both enzyme activities are catalyzed by the same molecular system. An increase in Vmax and activation energy of this enzyme were also found in DMD. Thus, Rega et al (1973) proposed that a change in the lipid domain could be responsible for these alterations. Ruitenbeek (1978) also proposed that Ca^{2+} metabolism in DMD RBC might be impaired as a consequence of altered Ca^{2+} transport.

1.1.1.3.3.2.2 LYMPHOCYTE CAPPING

There has been considerable interest in the capping of lymphocytes in patients with DMD. However, the results from different groups are once again contradictory. Recently, Horenstein and Emery (1983) together with previous reports (Pickard et al., 1978) reported a consistent failure of cap formation in lymphocytes from a large group of patients with DMD. It was proposed that this method might be valuable for carrier screening and prenatal detection of afflicted foetuses. In contrast, Sand and Harris (1979) found normal capping in most of the patients and carriers using fluoresceinate labelled $F(ab)_2$ fragment of anti-human immunoglobulin from rabbit or goat and with a

shorter incubation time for the capping process. Their findings are confirmed by several workers (Sybert et al.,1979).

1.1.1.3.3.2.3 CULTURED SKIN FIBROBLASTS (CSFs)

Roses et al (1980) studied the protein from solubilized whole CSFs: neither the distribution nor the quantity of individual coomassie blue-stained proteins is reproducibly different, although individual experiments may suggest minor variations. They also could demonstrate no changes in cell phosphoproteins labelled with sensitive isotopic techniques using the incorporation of [^{32}P] orthophosphate into living cell monolayers. DMD CSFs have a lower intracellular adhesiveness compared with normal CSFs when aggregated in a Couette viscometer. Collison efficiencies of 2.52 and 4.62, respectively were reported (Jones and Witkowski, 1983). It was also found that a very small proportion of DMD CSFs form large aggregates (Jones and Witkowski, 1981). Normal cell suspensions only rarely contained large aggregates but did contain many intermediate-size aggregates. These differences are not related to gross morphological differences between normal and DMD CSFs (Jones and Witkowski, 1979) and were not reflected in differences in intramembrane particle densities or distributions (Jones and Witkowski, 1983). In addition, membrane proteins and total proteins have also been studied (Burghes et al.,1982) using polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS). Regions of 73,000 to 68,000 and 48,000 molecular weight were decreased in DMD samples. These findings were found to be significant by both methods of statistical analysis (inter-experimental variation [termed "batch statistics"] and the student t-test). Regions of 175,000 and 53,000 molecular weight were significantly elevated in DMD preparations only by t-test, whereas a region of 32×10^3 molecular weight was only significantly elevated when

tested by batch statistics.

1.1.1.3.3.2.4 PLATELETS

It is surprising that platelets which are known to have functional similarities with muscle, especially with regard to Ca^{2+} kinetics, (Detwiler et al.,1978), have been infrequently investigated in neuromuscular diseases. The few existing studies indicate that platelet membrane abnormality can occur in muscular dystrophies (Yarom et al.,1983). A number of platelet defects including elevated Ca^{2+} content and surface membrane abnormalities were found in a few cases of DMD (Yarom et al.,1982). Recently, Yarom et al (1983) investigated 14 patients with muscular dystrophy and 20 suitable controls. In 4 DMD and one Limb-Girdle dystrophy the induction of aggregation by adrenaline and ADP was impaired. Electron microscopic and chemical examinations revealed an increased number of dense bodies, changed permeability and/or binding of cations and elevated intracellular Ca^{2+} in all the 9 cases of DMD while the 2 limb-girdle and 3 myotonic dystrophies were varied.

1.1.1.4 ADVANTAGES AND DISADVANTAGES OF USING FIBROBLASTS

The primary purpose of human tissue culture is to provide environmental conditions under which CSFs derived from an individual can be maintained outside of the donor (Freshney,1983). To allow the particular cell or cell population to grow and proliferate, the specific metabolic requirements of the CSFs must be satisfied by the constituents of the culture medium. Theoretically, this extracellular environment should closely match the physiological conditions found in the living host. It should provide a balanced salt solution, abundance of nutrients, proper temperature, and control of pH (Race, 1973). CSFs have been extensively used as a tool for studying human

genetic disorders. There are advantages and disadvantages in using these CSFs for such investigations. One reason CSFs are widely used is because they represent an easily accessible source from skin biopsy. For research purposes, using CSFs has many other advantages, such as the ability to easily control the environmental conditions required both for proliferation and for differentiation of the CSFs and the potential for production of large amounts of CSFs which allow a practically unlimited number of determinations. They can also be banked in liquid nitrogen (-70^{o}C) for further studies or shared with other investigators. They also form a homogenous population of monolayer CSFs (compared to a mixed cell population of a muscle cell culture) which *in vitro* resemble the normal CSFs in *in vivo* growth rate, maximum density in culture, and locomotory activity (Witkowski and Jones, 1981). Regarding the diagnostic aspects of human genetic diseases, fibroblast cells are a potential tool for the prenatal detection of disease in the foetus (Hug *et al.*,1973). During pregnancy fibroblast cells are relatively easily obtained from the amniotic fluid (removal by amniocentesis techniques, Emery, 1974) and can be cultured. It should always be kept in mind that culture conditions are artificial and subject to a number of pitfalls, such as infection by virus or mycoplasma (McGarrity *et al.*, 1978). This contamination is difficult to monitor and is a vital factor to further studies or culture. Besides that CSFs are also dramatically affected by their environment (culture medium). Since they are living, functional CSFs in a defined environment outside the body they are affected by (amongst other conditions), pH, temperature and culture milieu as well as passage number. Robbins *et al* (1971) studied seven human CSF lines derived from skin biopsies at passage numbers from 1 to 22, and reported the consistent accumulation of lysosomes as the passage number was increased. They also noted that, at later

passages, the lysosomes started to undergo degenerative changes. Lipetz and Cristafalo (1972) studied human CSFs ranging from the 16th passage to the 56th passage. They found an increase in the number of autophagocytic bodies (lysosomes), changes in mitochondrial structure, and increase in the invagination of the nucleus as the passage number increased. They noted that medium changes would produce a sharp decrease in the number of autophagocytic vacuoles. They suggested that the congestive engorgement of lysosomes plays an important role in the decrease in activity of cell populations at later passage number. It was also reported that, at later cell passages, there was an increase in the presence of microfilaments, and an increase in the prominence of the cisternal system of the cell. Lie et al (1973) were concerned with the effects of pH on the ultrastructure of human CSFs. They were able to demonstrate that an increased pH could increase the number of lysosomes in the CSFs. They suggested that this increase in cytoplasmic bodies may result from an inhibition of lysosomal function as the pH was increased. Douglas et al (1976) reported on the fine structure of normal human CSFs. They reported the presence of well-developed Golgi complexes, extensive areas of rough endoplasmic reticulum, and many free ribosomes in the cytoplasm. By changing the concentration of specific components of the medium, they were able to dramatically alter the ultrastructure of the CSFs, such that Golgi complexes were rarely observed, cisternae in the rough endoplasmic reticulum were reduced in number, and the number of free ribosomes in the cytoplasm was reduced. In order to minimise variation between disease and control lines, they can be matched for age, sex, race, and biopsy site. This latter parameter has recently been demonstrated to be of vital importance since a reported protein abnormalities in DMD CSFs (Rosenmann et al.,1982) was found to be due to differences in biopsy site between patients and controls (Thomson et al.,1983).

Growth stages in the culture can be matched by studying cell monolayers under conditions where, for example, growth rate is at a maximum (log phase). Another major disadvantage of using CSFs to investigate a disease, like DMD, where the defective gene is unidentified is that 1/3 of the recognised genetic defects in man are not expressed in CSF lines (Raivio and Seemiller, 1972).

1.2 LIPID COMPOSITION AND ORGANIZATION OF BIOLOGICAL MEMBRANES.

Study of membranes has recently become one of the most popular topics of basic and applied biochemical research. The importance of cell membrane alterations in disease states can be inferred from the general importance of membrane phenomena in normal reactions of the cell to extrinsic or intrinsic stimuli (physiological or pathological) which, of necessity, involve membrane interactions. It is probably safe to generalize that membrane alterations occur as a part of all disease processes. Understanding of cellular pathology therefore requires accurate knowledge of both the composition and orgnisation of biological membranes either in the normal or abnormal (disease) state.

1.2.1 MEMBRANE MODELS

The existence of membranes around living cells has been established for over a century due to the studies of Nageli who, in 1855, discovered that undamaged cells could adjust their volume in response to the osmotic strength of the surrounding medium. These studies were extended by Overton (1895) who observed that non-polar molecules more readily traversed the membranes of cells than polar compounds and on the basis of these studies proposed, for the first time, that cell membranes were lipid in nature. The development of ideas on membrane structure was advanced significantly by Gorter and

Grendel (1925) who, from studies on RBCs, first proposed the concept of a lipid-bilayer. The idea that proteins were associated with membranes was proposed ten years later by Danielli and Davson (1935) to account for the apparent discrepancy between the surface tension at membrane/water and oil/water interfaces. Since then, many different models have been proposed to explain membrane structure as well as the interaction between lipid and protein. At the moment only Singer and Nicolson's (1972) model (Fig. 1) accounts for most of the observed features of plasma membranes, and suggests that the membrane structure is a lipid bilayer (the structure of the major lipids of biological membranes is shown in Fig.2) with the polar groups exposed at the surface and the fatty acid chains occupying the interior of the bilayer. Some proteins penetrate into the hydrophobic region of the bilayer, while some polypeptides span the bilayer. The proteins, lipids and carbohydrates are asymmetrically distributed in the structure and together form a mobile, flexible, yet cohesive sheet.

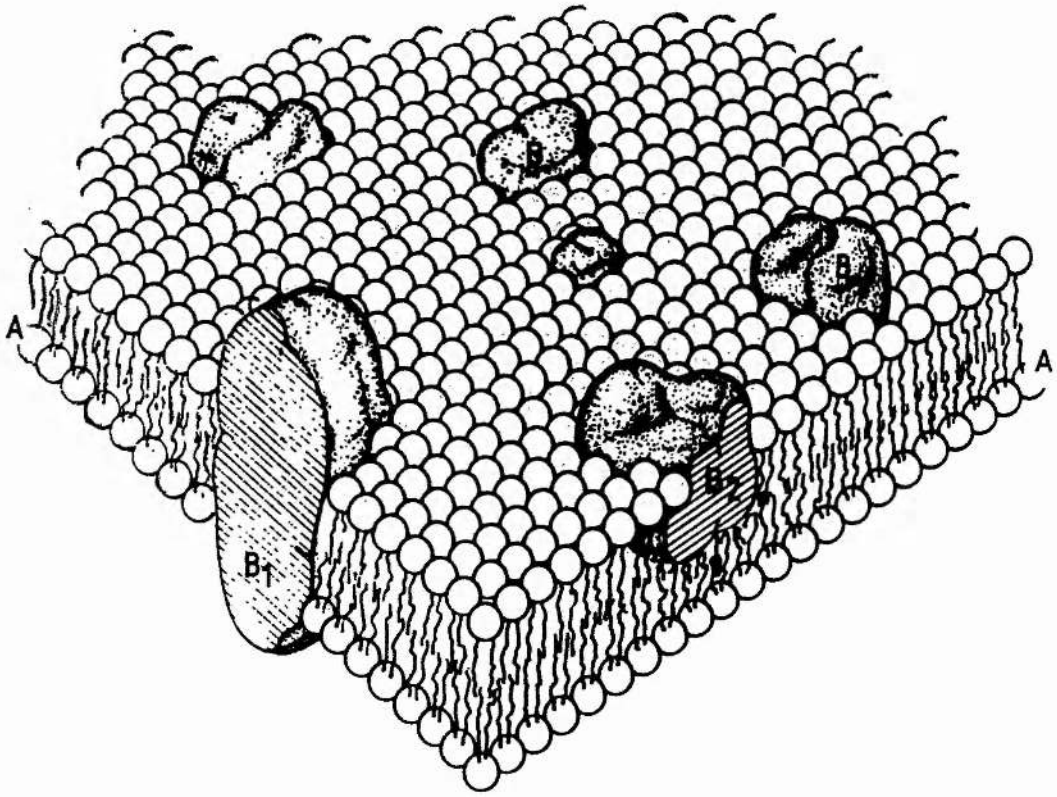
1.2.2 MEMBRANE LIPIDS IN DMD

1.2.2.1 MUSCLE

1.2.2.1.1 MUSCLE BIOPSIES

The total concentration of phospholipid was reported to be increased in DMD muscle plasma membrane (Kunze, 1977). An evaluation of the free fatty acids in the triglyceride fraction of muscle from DMD patients has also been reported (Pennington *et al.*,1966). A decrease in 16:0 and 22:6 and an increase in 16:1, 18:0 and 18:1 were reported. Two years later Takagi *et al* (1968) reported a significantly increased level of 18:1 and decreased 18:2 in PC in DMD muscle. Supporting those findings, Kunze *et al* (1975) found increased TG, with decreases in PC and PE in DMD muscle. Pearce *et al* (1981) also found that the SM content of muscle biopsies was increased, thus confirming previous findings by other groups (Kunze *et al.*,1975),

Figure 1. The fluid mosaic model of Singer and Nicolson (1972): the predominantly fluid lipid molecules (A) form the bilayer; proteins (B) may span (B_1) or rest in (B_2) the bilayer.



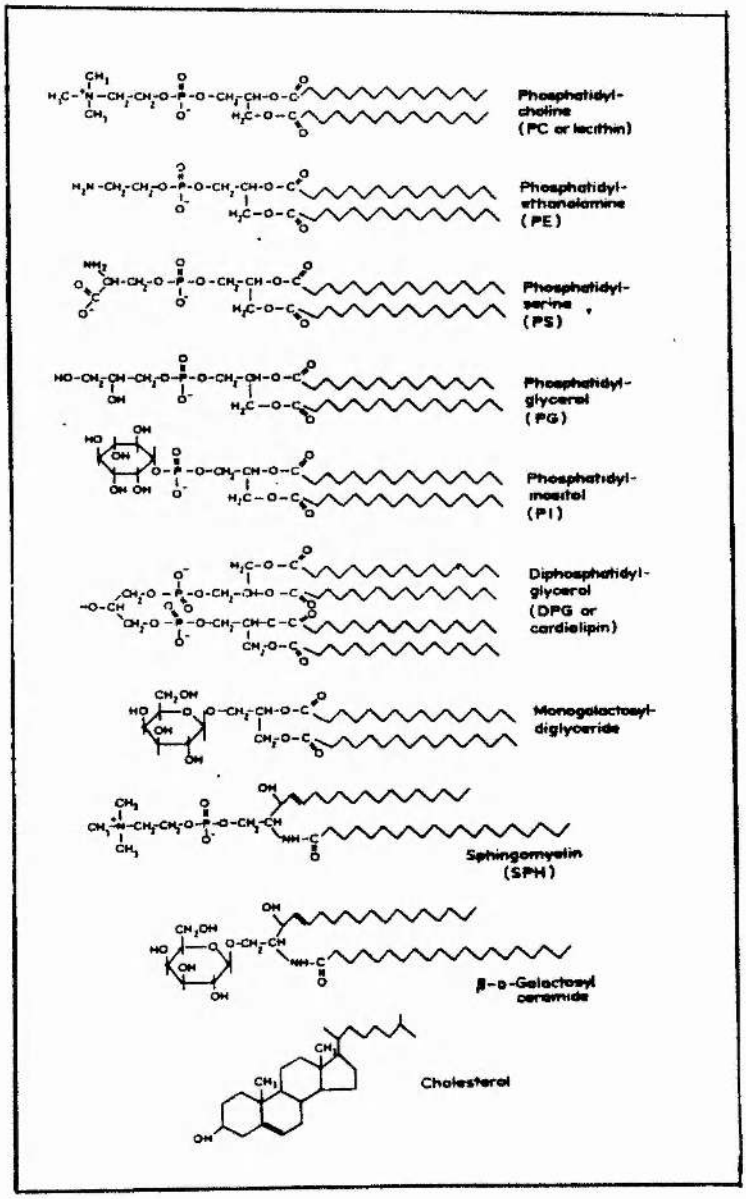


Figure 2. Lipids in biological membranes.

whilst that of PC was found to be decreased (Hughes and Takagi, 1971). Kunze et al (1970) proposed the decreased PE and increased lysoPC were the consequence of an enzyme defect in the disease. A similarity between immature muscle and DMD muscle was suggested by Hughes (1972) as he found an increase in total cholesterol and a decrease in choline plasmalogens in DMD muscle biopsies. Regarding fatty acid composition, different groups reported different results. Kunze et al (1975) found normal fatty acid moieties in TG, SM and PE of DMD muscle, but noted changes in the PC fraction. A decrease in 18:2 and a corresponding increase in 18:1 were found. Susheela (1968) reported a normal free fatty acid concentration in muscle biopsies of DMD patients. Hughes (1972) suggested that the fatty acid pattern of PC of DMD muscle was similar to that of neonatal muscle, in contrast, Takagi et al (1968) reported differences in DMD lipid composition from those of neonatal muscle. Clearly the results are contradictory between laboratories. These discrepancies could arise because of the heterogeneity of muscle biopsies with infiltrated fat and connective tissue as well as other variables such as age, activity, nutrition and location of the muscle.

1.2.2.1.2 CULTURED MUSCLE

Cultured muscle has distinct advantages over muscle biopsies since the possibility exists (see above) that muscle biopsies are contaminated by differing amounts of infiltrated adipose and connective tissue which may account for discrepant results as suggested by Pearce et al (1981). A markedly increased number of lipid droplets in DMD muscle explants compared with controls under identical tissue culture condition was reported (Bonsett et al.,1979). Bonsett et al (1979) also suggested an abnormality in fat metabolism in DMD skeletal muscle and/or other cell types. However,

up to the present time there are no published compositional studies in DMD cultured muscle.

1.2.2.2 RED BLOOD CELLS

Since a plausible unifying explanation for some or all of the many RBC abnormalities is altered composition and or organization of the lipid bilayer many studies of lipids have been carried out. Kunze et al (1973) reported a significant increase ($P<0.05$) in SM and a decrease ($P<0.05$) in a phospholipid fraction that was thought to be composed of PE and PS in DMD. Further, they found the fatty acid patterns of PE and SM were significantly altered in DMD. Specifically there was decreased 20:4 in PE while in the SM fraction 16:0, 20:4 and 18:2 were diminished and the percentage of 18:0 was elevated. On the other hand, Howland and Iyer (1977) and Ruitenbeek (1978) reported that the content of 16:1 in DMD is significantly decreased, raising the possibility that an alteration in TG metabolism may be present in DMD membranes. However, TG is such a small component of the total lipid raises doubts as to the significance of the finding for the disease. Proportions of fatty acids in corresponding fractions in the serum were not altered. Kalofoutis et al (1977) examined individual phospholipid classes and found a significant decrease in PC and increased LPC, SM and diphosphatidylglycerol. Other studies were unable to report any significant differences in SM (Howland and Iyer, 1977; Kobayashi et al.,1978; Koski et al., 1978; Ruitenbeek, 1979; McLaughlin and Engel, 1979). It was suggested that the discrepancy in the results was because Kunze et al (1975) and Kalofoutis et al (1977) did not take precautions to prevent autooxidation which could cause consequent selective loses of the more unsaturated species of lipid. In addition, to confirming the normal phospholipid class composition, Hunter et al (1983) suggested that the normal asymmetric organization

of membrane phospholipids may be deranged from studies using phospholipase A_2 from bee venom as a probe of glycerophospholipid organization. Phospholipase A_2 is the enzyme which catalyzes the hydrolysis of fatty acids in the 2-(beta) position, producing 2-lysophosphatides. The concentration of lysolecithin in RBC membrane is critical, since it is thought to be potent agent for induction of shape changes in RBC (Sato, 1973). A higher activity of endogenous phospholipase A_2 in DMD RBC as well as in MyD was reported (Iyer et al.,1976). Its found an elevation of the activity of 60% in DMD and as high as 242% in MyD.

1.2.2.3 CULTURED SKIN FIBROBLAST CELLS

So far only one group has published data on composition of DMD used CSFs (Kohlschutter et al.,1976). They claimed that there was no abnormality in neither phospholipid class distribution nor fatty acid composition of individual phospholipid classes in DMD CSFs. The normality of phospholipid class composition has also been confirmed in this laboratory (Carson, 1982).

1.2.2.4 ADIPOSE TISSUE

One of the well know indices of human muscular dystrophy is lipid accumulation (fat infiltration) within muscle fibres (Harriman and Reed, 1972) and gross fatty replacement of lost muscle (Walton and Gardner-Medwin, 1981). Goyle et al (1973) have revealed a significant increase in TG and decrease in phospholipids judged by cytochemical studies in dystrophic muscle in vitro. A simultaneous in vitro study of subcutaneous adipose tissue in patients at an advanced stage of muscular dystrophy and neurogenic atrophy revealed poor growth and a relatively less intense reaction for phospholipid (Goyle et al.,1973). Recently, Banerjee and Goyle (1983) again studied the

lipid of subcutaneous adipose tissue from normal and dystrophic human samples with particular reference to the fatty acid composition of neutral lipids and phospholipids. They found that the free fatty pattern did not show much change. TG, which together with cholesterol composed most of the neutral lipid fraction, showed significant altered in fatty acid contents by gas-liquid chromatography (GLC). There was a decrease in the total phospholipids, but an increase in proportion of SM and ethanolamine phosphoglycerides. Fatty acid composition of the different phospholipid classes analyzed by GLC showed significant changes. In choline phosphoglyceride, a large decrease was found in 16:0, while 18:1 increased significantly in DMD compared with controls.

1.3 MEMBRANE LIPID PEROXIDATION

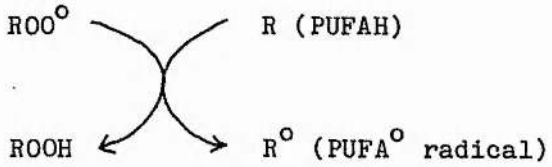
1.3.1 INTRODUCTION

The role of membrane lipid peroxidation as a factor in various pathological states such as chlorinated hydrocarbon hepatotoxicity, ethanol-induced liver injury, air pollution by oxidants, atherosclerosis, cancer, sickle cell anaemia and major and minor thallassemia has been a subject of intense interest and controversy in recent years. The possibility that lipid peroxidation may be a basic mechanism of cell damage for a wide spectrum of diseases has been clearly suggested from an increasing number of animal and human studies. The purpose of this section is briefly to point out several suggested mechanisms whereby free radicals may originate and initiate lipid peroxidation reactions in a biological system and also to illustrate how a biological system copes with both endogenously and exogenously induced membrane lipid peroxidation. Several techniques to measure lipid peroxidation products will also be described.

1.3.2 CHEMISTRY OF PUFA PEROXIDATION

1.3.2.1 GENERAL FEATURES

Tappel (1973) broadly defined lipid peroxidation as the oxidative deterioration of PUFAs. Peroxidation of lipids involves the reaction of free radicals with PUFAs to form lipid free radicals which react with O_2 to form semi-stable peroxy radicals which then promote free radical chain oxidations (Tappel, 1973). The free radical chain reaction proceeds in three distinct steps (Pryor, 1973). The first is the "initiation" process by which the lipid radicals are generated. The second is a series of "propagation" reactions in which the number of free radicals is conserved or increased as the peroxidation reactions proceed. Finally, there is a series of "termination" reactions by which free radicals are destroyed. The three steps of lipid peroxidation are depicted below in a simplified scheme (Fig.3). The initiation of lipid peroxidation occurs where a hydrogen atom is abstracted from an unsaturated fatty acid and a free radical is formed ($PUFA^{\circ}$); propagation occurs when the fatty acid peroxyl radical ($PUFAOO^{\circ}$) abstracts a hydrogen from another PUFA (PUFAH), itself becoming a hydroperoxide, and generating another fatty acid peroxyl radical ($PUFAOO^{\circ}$) as shown below:



and chain termination occurs where two radicals meet and end the progression, or where a free radical scavenging antioxidant, e.g. alpha-tocopherol, provides a hydrogen atom to block the free radical abstraction from another PUFA.

1.3.2.2 INITIATION OF LIPID PEROXIDATION

The endogenous concentration of O_2 within most tissues is

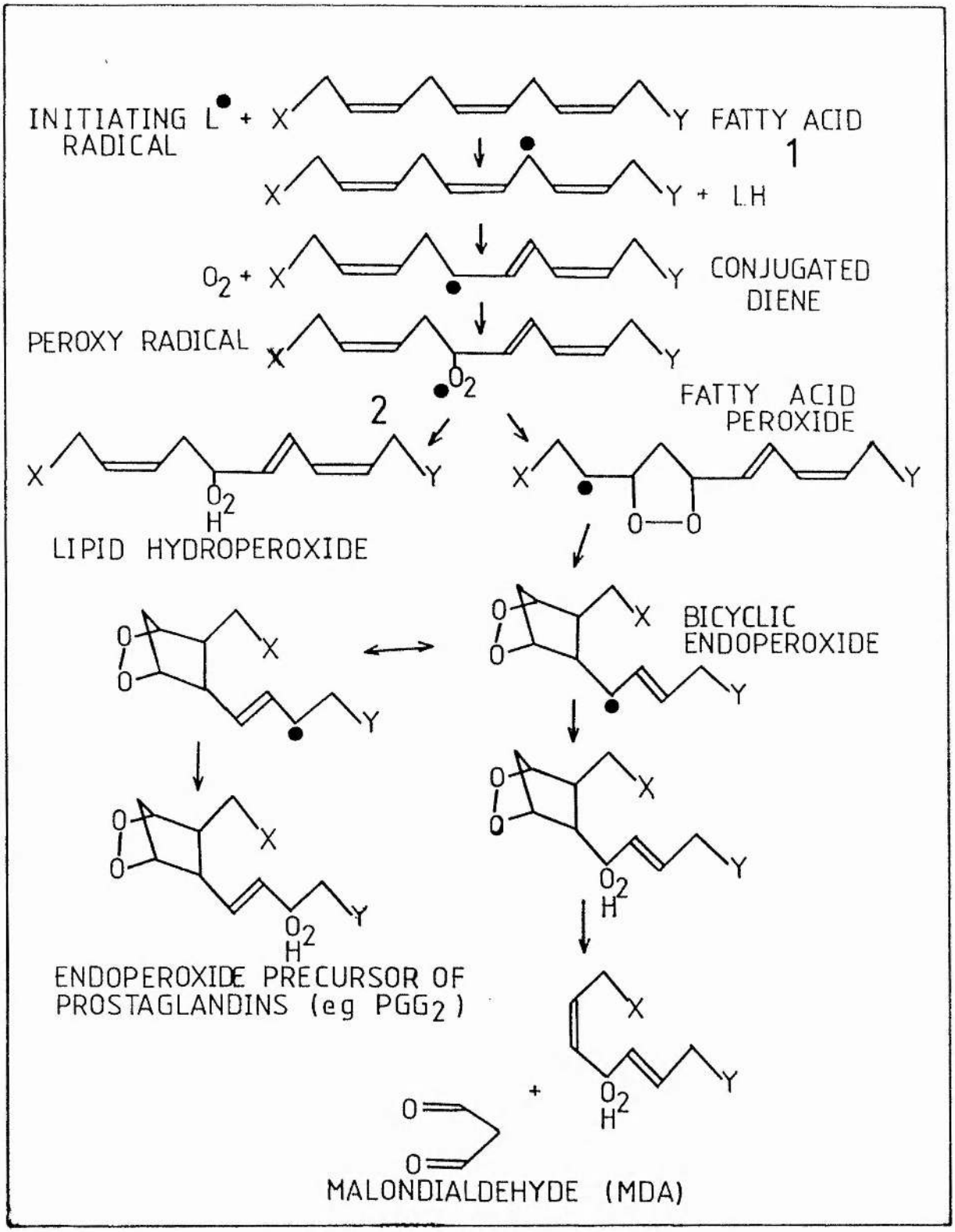
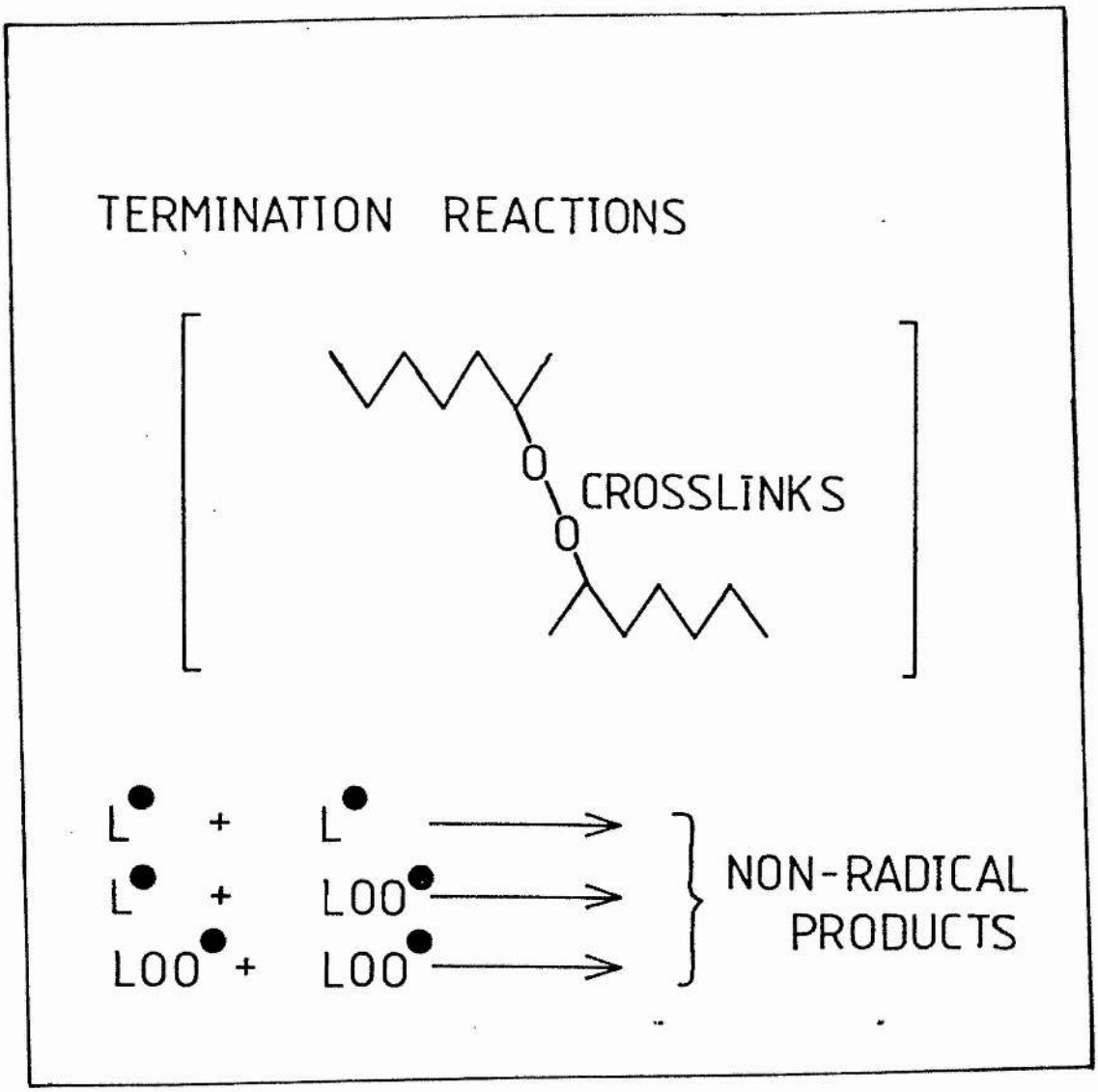


Figure 3. Mechanism for conversion of methyl linolenate to conjugated hydroperoxides and prostaglandin-like endoperoxides, and the further conversion of these prostaglandin-like endoperoxides to form the TBA-reactive material, i.e. MDA. X=$(CH_2)_6CO_2CH_3$, Y=CH_3 (From Pryor and Stanley, Lipids 11(5):371; 1976; and Pryor et al.,J.Org.Chem. 40(24):3616; 1979).

Figure 3. (continued)



thought to be unsufficient to facilitate autocatalytic peroxidation of membrane lipids (McCay and Poyer, 1976). However, recent evidence has indicated that a number of biological systems can convert O_2 to potentially toxic species of O_2 radicals such as singlet oxygen ($^1O_2^*$) and superoxide radical (O_2^{-o}). Another important aspect is the dismutation of O_2^{-o} resulting in the production of another potentially toxic material, H_2O_2. Finally, homolytic fission of the O-O bond in H_2O_2 produces two hydroxyl radicals, ^oOH. Homolysis can be achieved by heat or ionising radiation and in the presence of an iron (II) salt (Halliwell and Gutteridge, 1984). These various forms of O_2 radicals, which are produced from endogenous oxidation reactions as well as oxidation of xenobiotics, are capable of either directly or indirectly initiating peroxidation.

The role of O_2^{-o} in the initiation of peroxidation reactions is currently subject of extensive investigation. O_2^{-o} is produced in many endogenous biological reactions; such as autooxidations of reduced flavins, hydroquinones and catecholamines, and from the aerobic actions of enzymes such as xanthine oxidase, NADPH oxidase, aldehyde oxidase and several flavin dehydrogenase (McCay et al.,1976). In addition, it has been proposed that the toxicity of several chemicals is mediated through the generation of O_2^{-o}. The O_2^{-o} per se, does not appear to be directly involved in the initiation of lipid peroxidation (McCay et al.,1976) It should be noticed that dismutation of O_2^{-o} results in the production of another potentially toxic species, H_2O_2. In 1934 Haber and Weiss proposed the formation of ^oOH (from interaction of O_2^{-o} with H_2O_2), which is an extremely potent initiator of peroxidation reactions, by the following reaction:

$$O_2^{-o} + H_2O_2 \text{ ---------> } {}^oOH + OH^- + O_2$$

An enzymic system which induces lipid peroxidation associated with the NADPH oxidase system, (NADPH-dependent lipid peroxidation)

was first described by Hochstein and Ernster (1963). It appears that concomitant membrane lipid peroxidation is an inevitable consequence of the operation of the NADPH oxidase system which operates in microsomes in e.g.: hydroxylation of steroids and xenobiotics. The requirements of the NADPH oxidase system include NADPH oxidase, iron (II) salt, and O_2. The proposed mechanism of NADPH-induced peroxidation (Bidlack and Tappel, 1972) is shown in Fig.4a. Generally, it involves catalytic reduction of ferric iron to ferrous iron and the subsequent cleavage of pre-formed hydroperoxides, accompanied by Fe^{2+} to Fe^{3+} oxidation, to produce free radical initiators of the peroxidation process. As indicated this process has been shown to operate *in vivo* but it is also used extensively as a model system for lipid peroxidation *in vitro*.

The O_2^{-o} may also initiate lipid peroxidation through the intermediate formation of $^1O_2^*$. $^1O_2^*$ has been demonstrated in several *in vitro* systems to rapidly react with PUFAs to form LOOHs (Anderson *et al.*,1974). Based upon the observation that NADPH-oxidase-catalyzed peroxidation of linolenate was inhibited by SOD, catalase (CAT), and $^1O_2^*$ scavengers, Kellogg and Fridovich (1975) proposed that the Haber-Weiss reaction liberates $^1O_2^*$ as follows:

$$O_2^{-o} + H_2O_2 \longrightarrow {}^oOH + OH^- + {}^1O_2^*$$

It has also been shown that iron and ascorbate stimulate peroxidation in mitochondrial suspensions as well as microsomal preparations (Wills, 1972; Fujita, 1972) (Fig.4b). Unlike NADPH-induced lipid peroxidation, this process is non-enzymic since it can be shown to occur in suspensions which have been heat inactivated. The administration of ascorbic acid along with iron greatly increases lipid peroxidation as compared to iron alone. Wills has reported that rats injected with iron plus ascorbic acid show a 74% increase in hepatic microsomal lipid peroxidation as compared with

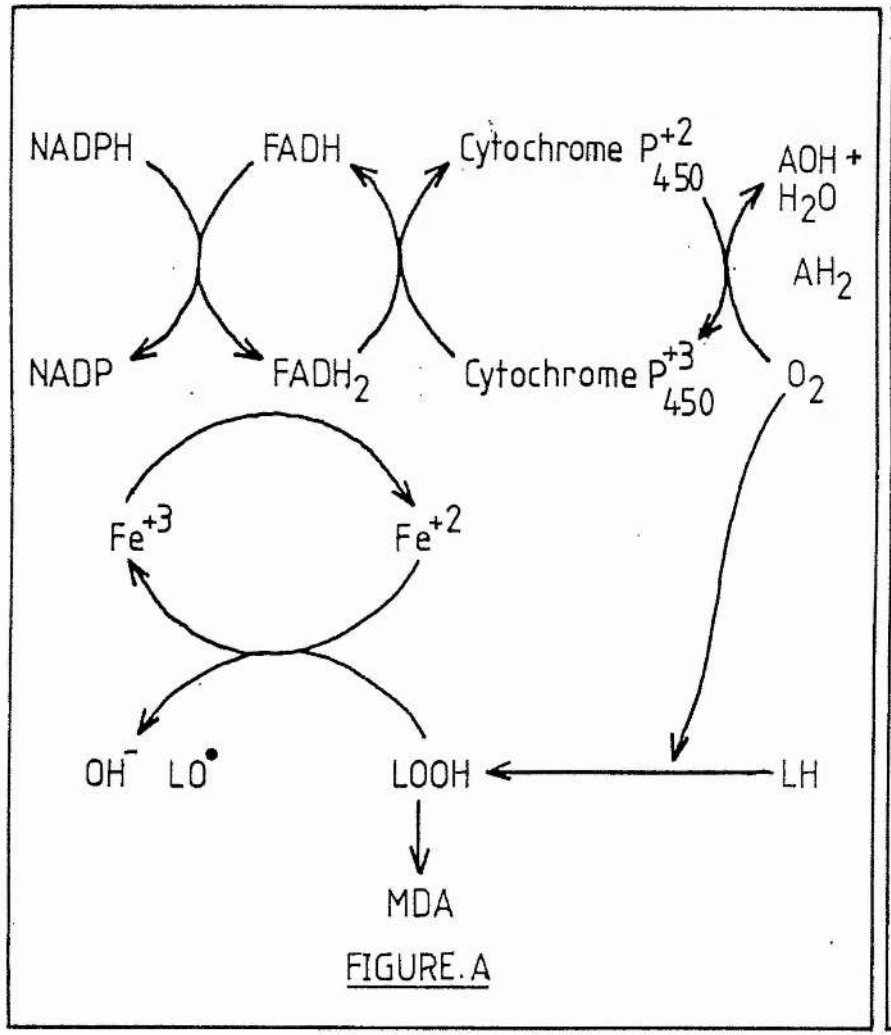


FIGURE.A

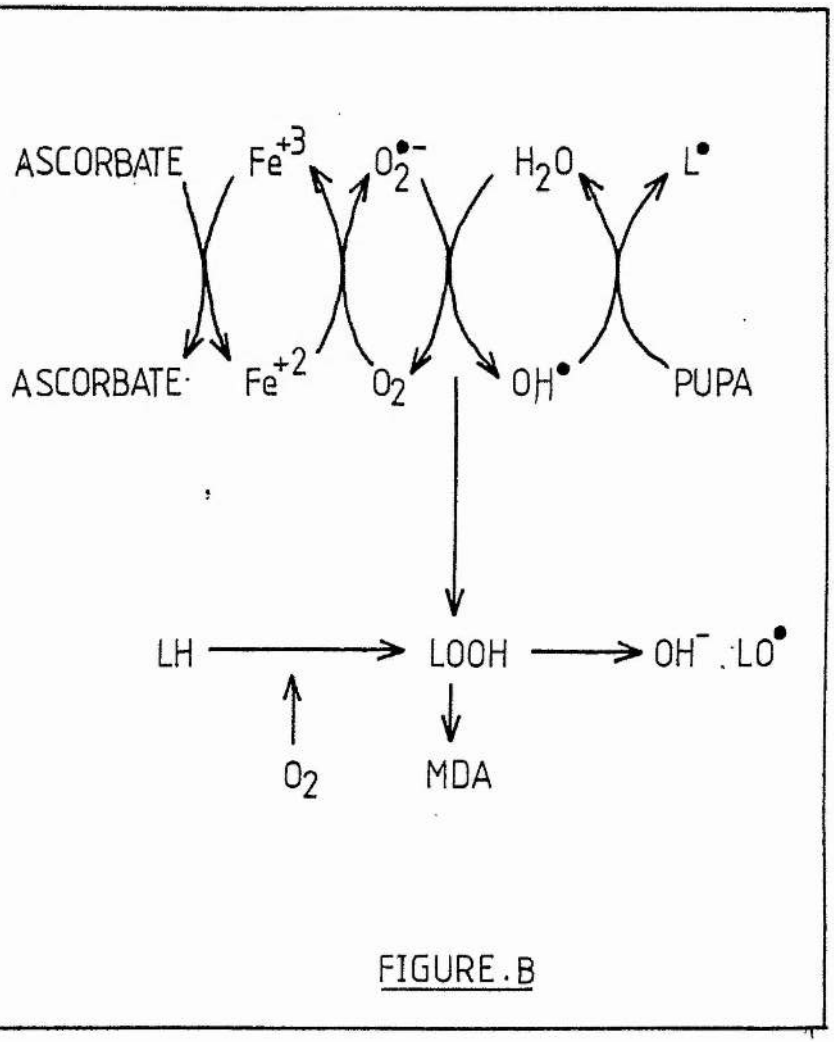


FIGURE.B

Figure 4. Suggested schemes for (A) enzymic and (B) nonenzymic-induced lipid peroxidation.

a 14% increase for iron administration only. Ascorbate could act as a reducing agent to maintain the iron in the ferrous state. Additional free radical lipid peroxidation initiators will be produced by enhanced cleavage of hydroperoxide (prompted by iron in ferrous state) as occurs in enzymic induction of lipid peroxidation. As for NADPH oxidase system, ascorbate/Fe^{3+}, in addition to stimulating *in vivo* lipid peroxidation has also been much used as an *in vitro* model for lipid peroxidation induction.

1.3.2.3 FURTHER REACTIONS LIPID PEROXIDATION PRODUCTS

Lipid peroxidation of PUFAs in biological tissues/membranes results in the formation of free radical intermediates and carbonyl compounds as by-products (Lillard and Day, 1965) (Fig.5). Reaction of fatty acid conjugated diene (CD) with O_2 yields the corresponding peroxyl radical ($PUFAOO^{\circ}$) and chain propagation ensues, leading to ultimately to degradation of the lipid to wide range of products including aldehydes (e.g. MDA) and hydrocarbon gases such as pentane and ethane (stable products). In contrast, free fatty peroxyl radical are able to interact each other leading to membrane phosholipid damaged at the end as well as other components (proteins, SH-membrane-bound enzymes) as result of lipid-lipid and lipid-protein cross-linked, respectively (Ingold, 1969).

Malonaldehyde (malondialdehyde-MDA) is one of the complex mixture of carbonyl compounds (Dahle *et al.*,1962). Several other carbonyl compounds are n-alkanals (propanal, butanal pentanal, hexanal), alk-2-enals (nonenal, lindecenal), alk-2,4-dienals (heptadienal and nonadienal), and 4-Hydroxyalk-2-enals (Hydroxyctenal, Hydroxynonenal, Hydroxyundecenal) (Esterbauer *et al.*,1982; 1984). Relative proportion differ with lipid composition of peroxidised membrane. The greater the degree of fatty acid unsaturation, the

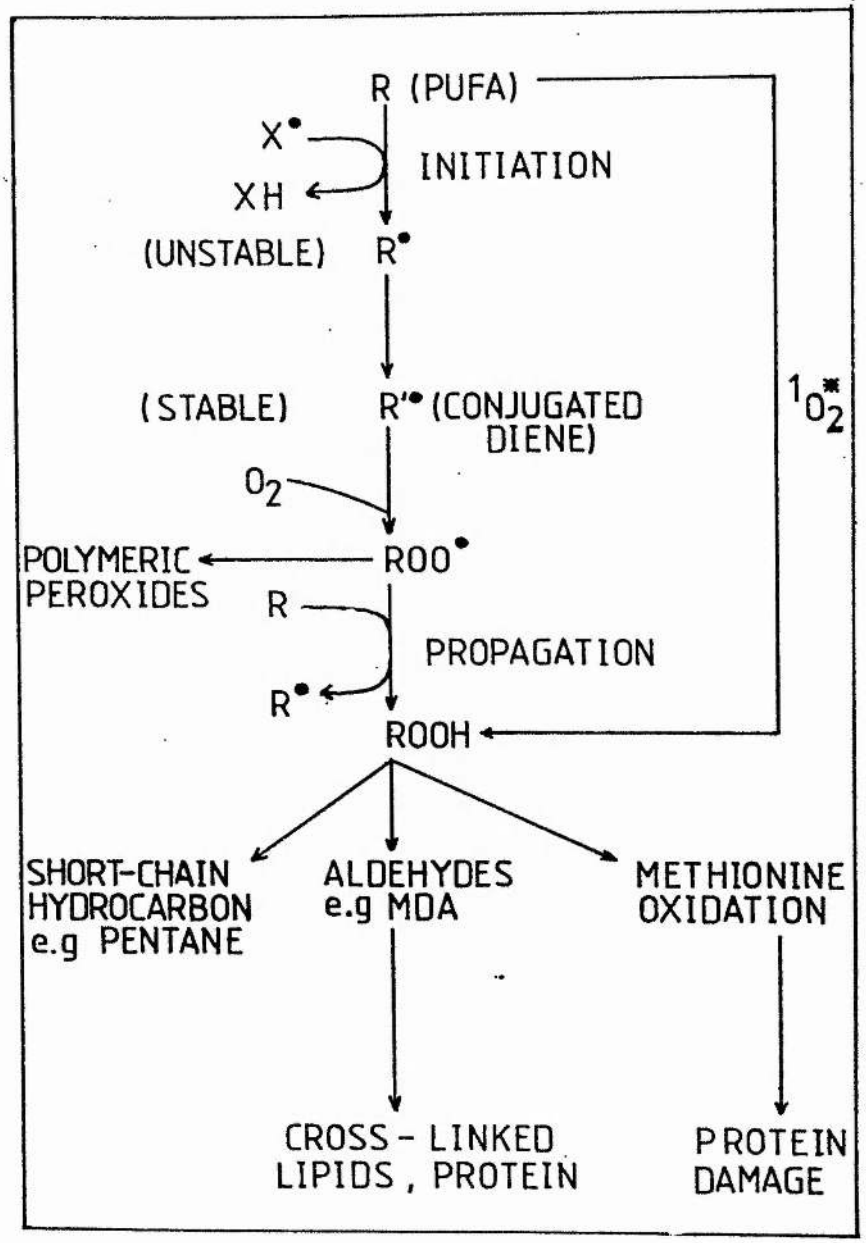


Figure 5. Simplified scheme for the polyunsaturated fatty acids (PUFA) lipid production products.

faster the rate of formation and the greater the amount of MDA formed. MDA is a very reactive bifunctional material. It was shown to react with and cross-link amino compounds by Crawford et al (1967). This reaction damaged proteins (especially SH-enzymes) and nucleic acids (DNA,RNA). Chio and Tappel (1969) have shown that concomitant with the loss of protein and enzyme activity was the appearance of a fluorescent chromophore. The fluorescent chromophore, R-N=CH-CH=CH-NH-R' develops from the cross-linking reaction of MDA with many biologically important amines, including RNA, DNA, protein, enzymes and phospholipids (Tappel, 1973). These fluorescent materials are identical with the "age" pigments lipofuscin and ceroid pigments which have been recognised histologically for a long time as signs of tissue ageing or damage. Because there pigments are an end product of lipid peroxidation and are not easily removed from cells, they represent a cumulative index of the amount of lipid peroxidation which has occurred. The major damage sites are the subcellular organelle membranes.

Further, reaction of methionine with $^1O_2^*$ (Hiller et al.,1981) in the methionine oxidation pathway yields its sulfoxide with flavins, under some conditions, methional is the product (Foote, 1981). The degradation of methionine by such oxidation is responsible for the the loss of activity of several important enzymes, altered or damaged proteins and ultimately damage to the whole cell especially the plasma membrane.

1.3.3 MEASUREMENT OF LIPID PEROXIDATION

Despite their wide application in in vitro studies, U.V assay and TBA analysis may not be entirely suitable for absolute in vivo measurement. Products of lipid peroxidation may exist at very low concentration in vivo. CD and MDA are unstable products and known to

be rapidly metabolized *in vivo* (Trombly *et al.*,1975) as well as to react with tissue components (Chio and Tappel, 1969), thus making *in vivo* measurements with this assay tenous at best. This problem may be of particular important in situations where *in vivo* lipid peroxidation is proceeding at rates slightly above endogenous peroxidation rates, such as might occur in disease states or with repeated low dose exposures to peroxidation-initiating xenobiotics. Therefore, the failure to detect CD and TBA-reacting substances in an *in vivo* experiment cannot be regarded as evidence for the absence of lipid peroxidation tissue damage.

Besides low concentration and transient nature of intermediates, the multiplicity of products is also of great importance to the measurement of lipid peroxidation. For example, it has been shown that autoxidized linoleic acid (18:2) contains at least nine breakdown products that give a positive response in TBA reaction when studied on TLC plates (Gutteridge *et al.*,1974). As a result, whether the TBA reaction is performed on biological samples it is demonstrating principally MDA or "MDA-like" substances, or decomposition products that arise during the heating stage of the reaction of the tissue with TBA. The specificity of the TBA reaction for MDA has been questioned several times (Slater, 1972; Esterbauer, 1984). Several techniques for measurement of lipid peroxidation have been applied. Fig. 6 is a simplified scheme showing some of these.

1.3.3.1 CONJUGATED DIENE (U.V ABSORPTION)

CDs are formed in the initiation reactions of PUFA peroxidation and exhibit spectra characterized by an intense absorption at 233nm (U.V absorption) and a shoulder due to ketone dienes in the region of 260-280nm (Holman, 1954). This method has been used satisfactorily for the non-specific demonstration of *in vivo* lipid peroxidation (Recknagel and Ghoshal, 1966). However, later, Waller and Recknagel

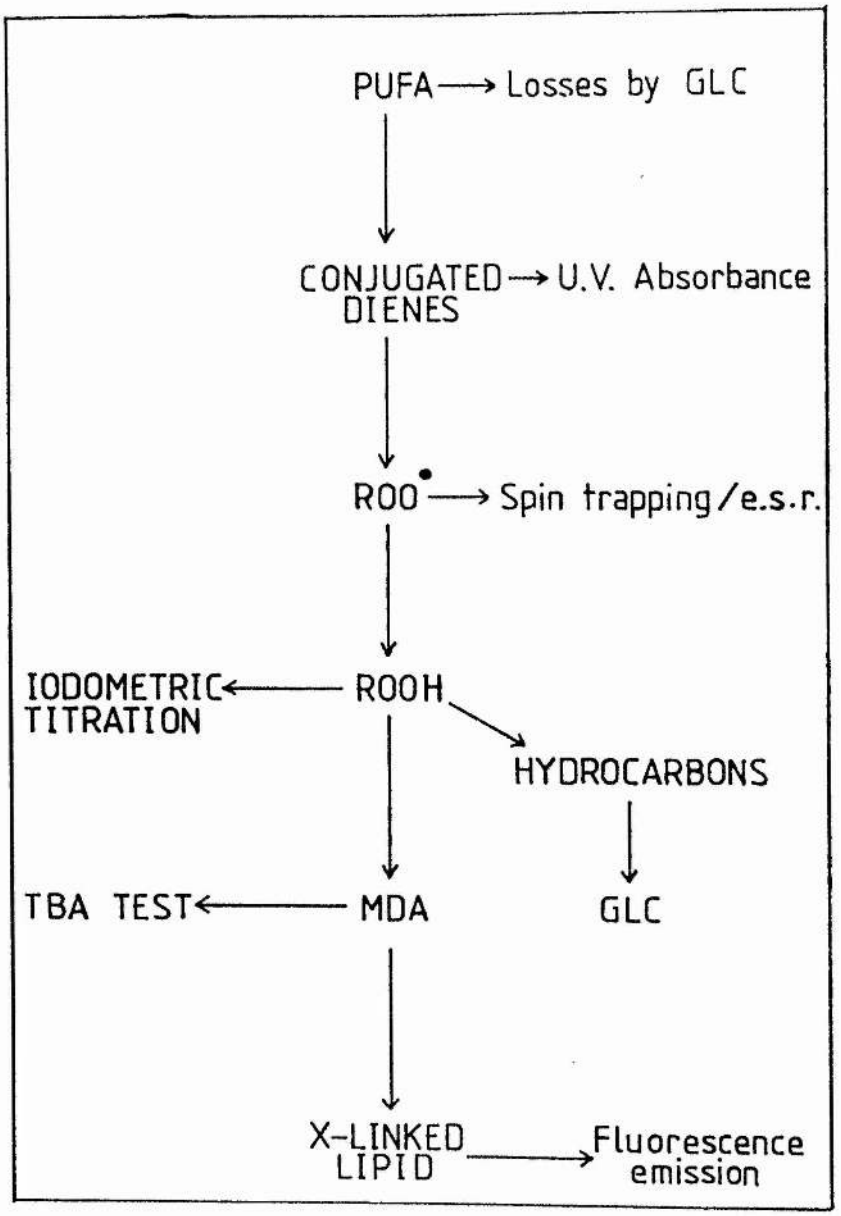


Figure 6. Summary of the direct and indirect methods available for the detection and measurement of lipid peroxidation.

(1977) described a method for specific quantitative analysis *in vivo*, utilizing tetracyanoethylene-^{14}C in a Diels-Alder condensation reaction to trap CDs. Thus, this method may prove to be an additional valuable tool for the study of the pathology of *in vivo* lipid peroxidation.

1.3.3.2 MDA ASSAY

Several methodologies have been developed which permit the measurement of lipid peroxidation occurring in biological systems. Among them, the thiobarbituric acid (TBA) test is probably the most commonly used method of measuring lipid peroxidation in pure lipids and incubated tissue suspensions owing to its convenience and high sensitivity. The TBA reacting substance is generally thought to be MDA (Yu and Sinnhuber, 1964; Bus and Gibson, 1979). Usually, the TBA test involves measuring the red-pink solution absorption maximum at 532nm. Red chromogen is formed due to condensation of TBA with MDA when boiled (100°C) in acid (15-45 minutes) mixture. Regarding reaction of TBA with other carbonyl compounds, Asakawa and Matsushita (1980) reported that, although alk-2,4-dienals (heptadienal and nonadienal) react positively with TBA and formed the red pigment, this is probably due to further oxidation of these alk-2,4-dienals to MDA and as suggested by the earlier findings (Patton and Kurtz, 1955). Further, Esterbauer (1982) found that major carbonyl products of peroxidation (n-alkanals and 4-hydroxyalk-2-enals) gave much smaller molar absorption coefficients than found with MDA. Sinnhuber and Yu (1958) crystallised the TBA chromogen derived from rancid salmon oil and found it was identical with an MDA-TBA complex in which two TBA molecules are linked by one of the dialdehydes. Formation of MDA and the TBA-MDA adduct under the conditions of the TBA test are shown in Fig.7., which also illustrates the important point that intermediates

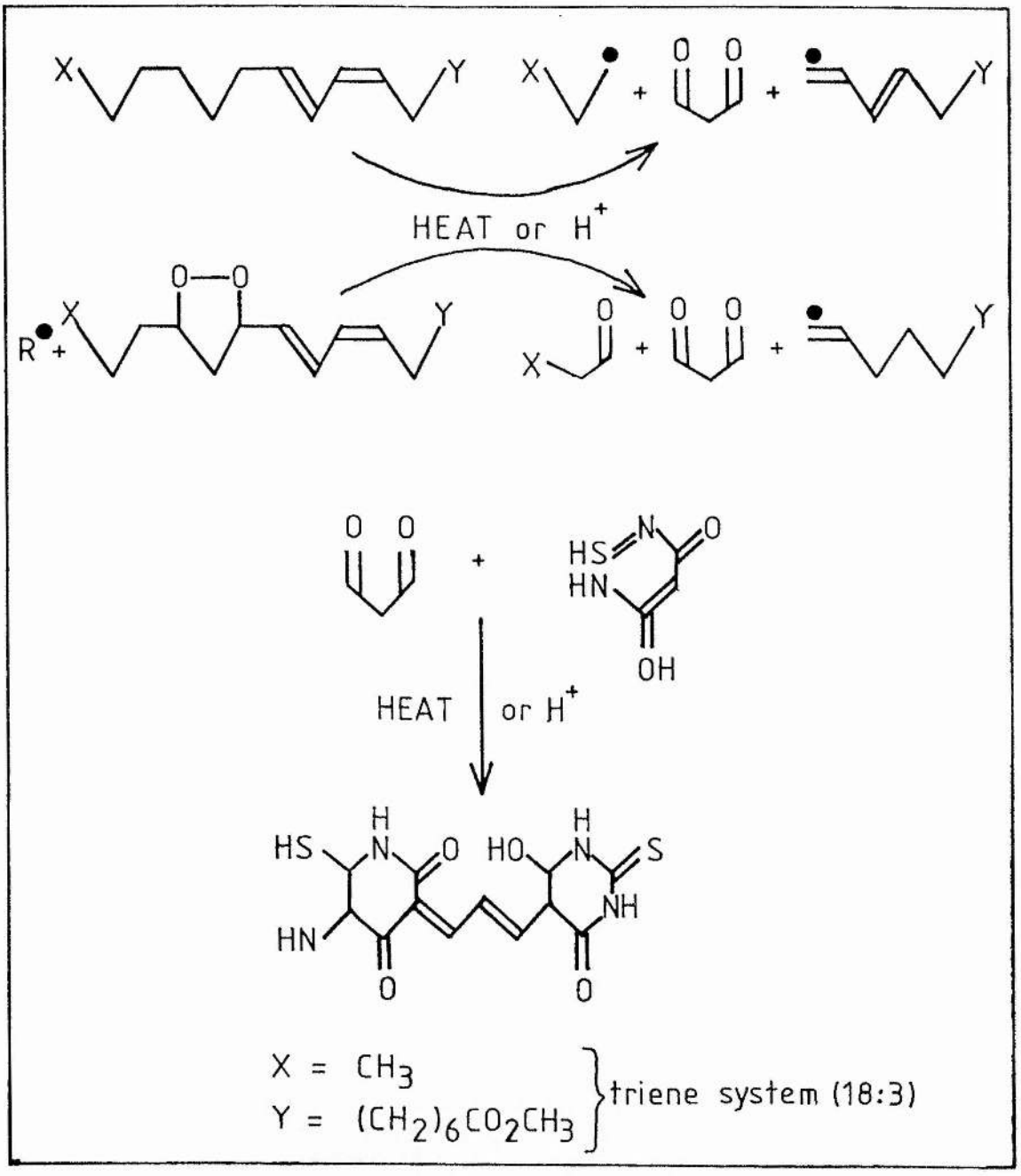


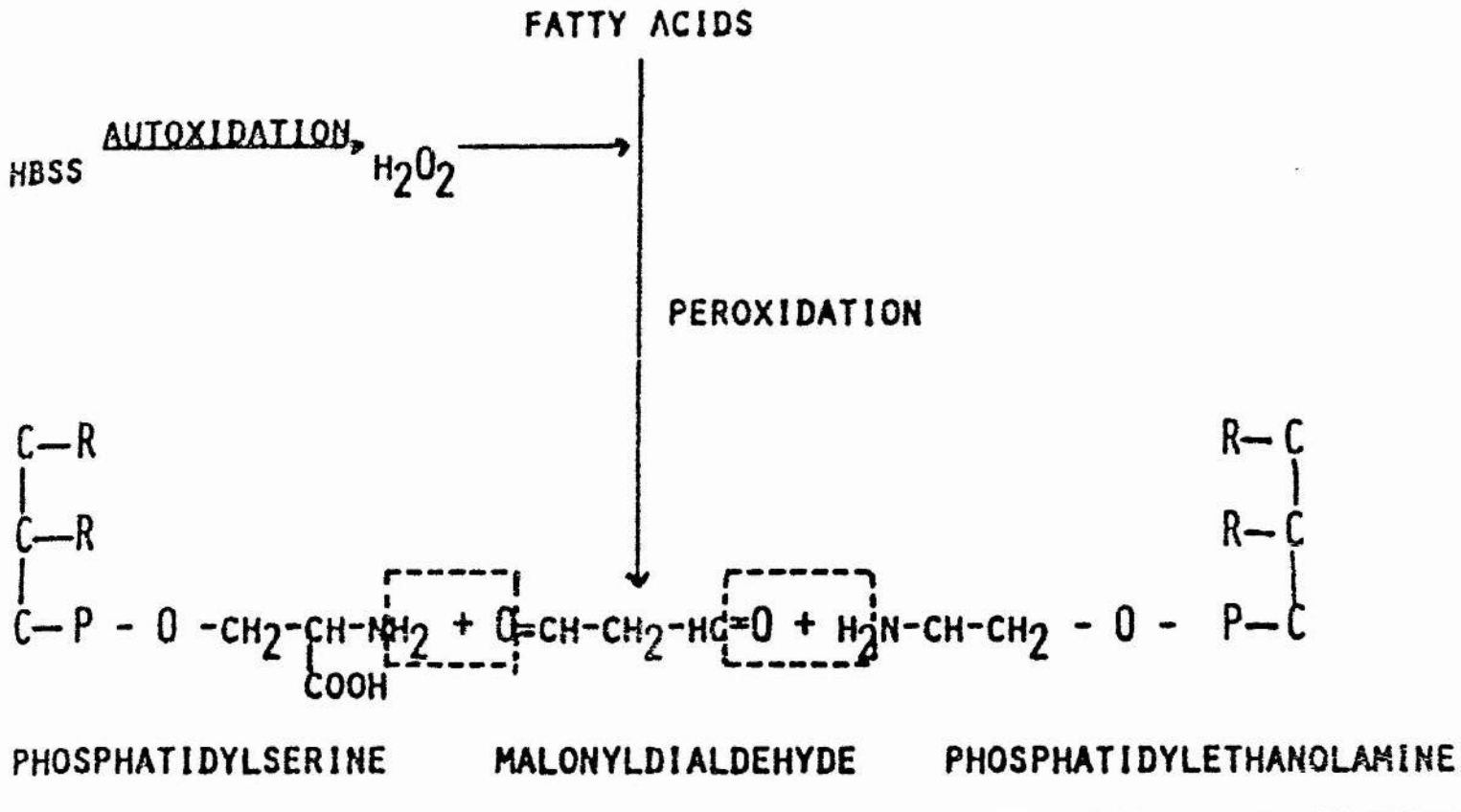
Figure 7. Formation of malondialdehyde (MDA) and the TBA-MDA under the condition of the thiobarbituric acid (TBA) test (From Pryor and Stanley, Lipids 11(5):371; 1976).

in the process of lipid peroxidation will also decompose under the conditions of the test to liberate MDA i.e. the TBA test does not usually measure free MDA unless a prior lipid extraction step is performed.

1.3.3.3 FLUORESCENT PRODUCTS

Since MDA itself is a very reactive material and is known to make cross-linkages with proteins and other cellular constituentes containing-NH_2 groups, (e.g. phospholipid such as PE and PS)(Fig.8) to form fluorescent products, the detection of lipid peroxidation in biological tissues by fluorescence has been used to quantity the process and is found to be 10 to 100 times more sensitive than the TBA test (Trombly and Tappel, 1975). These pigments (general structure R-N=CH-CH=CH-NH-R') have an excitation maximum at 370-470nm and a fluorescence maximum at 450-470nm. They are fall into two solubility classes: lipid soluble and water soluble. The water soluble forms are probably derived from amine-containing molecules such as amino acids, proteins, nucleic acids, etc, which have reacted with carbonyls derived from peroxidizing PUFAs. Aqueous extracts of peroxidizing tissues contain other fluorescent water soluble compounds such as flavins, reduced pyridine nucleotides, proteins as well as carbohydrate origin, which are difficult to remove. Because of these interfering compounds, the use of aqueous extracts is limited mainly to in vitro model systems where interfering fluorescent compounds are excluded or are present at low levels (Fletcher et al.,1973). Lipid extracts give lipid-soluble fluorescent derived only from lipid peroxidizing systems. They have fluorescence absorption maxima in the 420-470nm region and excitation maxima in the 340-370nm region. Lipid extracts from membranes have two types of interfering fluorescent compounds, retinol and flavins. Retinol has fluorescence in the 475nm

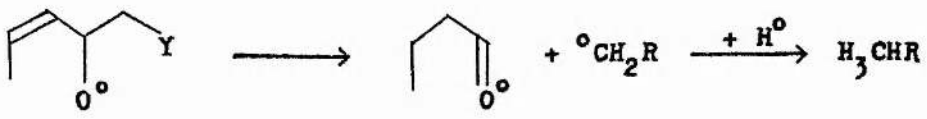
Figure 8. Schema showing Schiff's base adduct formation between phosphatidylserine, phosphatidylethanolamine, and malondialdehyde. (From Tappel, 1972).



region and excitation at 280, 350 and 450nm regions. The flavin compounds (probably lipoflavoproteins) are easily removed from the chloroform layer extracts by the water-wash step of the extraction procedure. Retinol is a fat soluble compound that is not removed during the water-wash step. An effective way to remove retinol fluorescence is to expose the chloroform-rich layer extract to high-density U.V for 30 seconds. However, in many biological systems the concentration of retinol is sufficiently low as to give negligible interference.

1.3.3.4 HYDROCARBON GASES

Several reports have appeared indicating the usefulness of the measurement of these gases as an index of lipid peroxidation in biological systems (Dillard et al.,1978). Evolution of hydrocarbons in vivo as a results of lipid peroxidation was first reported by Riley et al., (1974) who found that hydrocarbon gases of lower molecular weight were released upon treatment of mice with CCl_4. Evans et al (1967) visualised a general route for the formation of these gases as shown below:



Hydroperoxide decomposition to LO° the key step in the proposed scheme which is followed by beta-scission and hydrogen abstraction resulting in the formation of hydrocarbon gases. Beta-scission of the LO° is a well known process (Tappel, 1975) and involves unpairing of electrons in the bond located beta to the free radical. The fact that, in biological systems, transition metals, particularly the iron and copper catalysts are present mostly in higher oxidation states, which help to form these gases in relatively greater amounts, supports the

above mechanism.

This method is also faced several disadvantages. Concentration of hydrocarbon gases are very low. Thus, it may not easy to monitor by GLC. Secondly, determination of gases might be contaminated by environmental pollution especially from automobile exhaust (smoke) and industries. Finally, it may also be necessary to prohibit the lighting of bunsen burners in a laboratory, as they are source of hydrocarbons (Cohen, 1979).

1.3.3.5 LOSS OF POLYUNSATURATED FATTY ACIDS (GLC)

This method involves the determination of total fatty acid composition of the tissue lipid by GLC before and after lipid peroxidation. It was first developed by May and McCay (1968) who reported the loss of PUFA moieties as a good index for the detection and measurement of lipid peroxidation. A major advantage of this over other methods is that it involves a direct analysis of the tissue lipids themselves as opposed to the detection of products resulting from peroxidation. In this respect, this method provides one of the most direct methods for the detection and measuring the extent of lipid peroxidation in biological systems. However, it is relatively insensitive and subject to error due to losses of PUFA during handling and analysis.

1.4 CELLULAR PATHOLOGY DUE TO LIPID PEROXIDATION

During lipid peroxidation, a saturated carbon no longer separates the two carbons with unsaturated bonds and this is referred to as conjugation; RO°, are present and react to form peroxides, ROOR, thereby linking two adjacent fatty acids in an abnormal bond; mobile $^{\circ}OH$ are formed as the result of hydroperoxide scission; hydrogens are then abstracted by $^{\circ}OH$, possibly from neighboring lipid and protein

molecules, resulting in lipid-protein, lipid-lipid and protein-protein cross-linking (Menzel, 1976) (Fig.9). Abstracted hydrogens react with ^{o}OH to form water in the hydrophobic midzone; fragmentation of fatty acid tails occurs with eventual production of negatively charged carboxylic acid groups. These interactions sufficiently alter the lipid environment to affect activity and structure of membrane-bound enzymes. Fig. 10 shows a schematic drawing of some of the various types of molecular damage to biomembrane components (e.g. membrane-bound enzymes) as a result of the propagation of peroxidation of the PUFA in the phospholipids. The SH-enzymes are most susceptible to inactivation by lipid peroxidation (Chio and Tappel, 1969), which may occur either by an alteration of membrane lipid structure and organization by cross-linking reactions or by SH-group oxidation. Thus, the cellular pathology of lipid peroxidation at the membrane level is exceedingly complex and involves not only the unsaturated lipids but also the many different proteins that are an integral part of membranes. Rouser et al (1968) reported that a high proportion of PUFAs were found in mitochondria and SR membranes. Consequently, these membranes are highly susceptible to lipid peroxidation damage. Beside that, some of the most potent catalysts involved in lipid peroxidation, coordinated iron and hemeproteins, are found in close association with these membranes (Tappel, 1973). Other examples of cell injury associated with the occurrence of lipid peroxidation include RBC hemolysis in vitamin E-deficiency (Brownlee et al.,1977), microsomal membrane enzyme activity leading to free radical disruption of the microsomal membrane (Archakov and Kurzina, 1973) and the breakdown of lysosomal membranes (Wills and Wilkinson, 1966) with resultant intracellular release of proteolytic enzymes, which in turn initiate random hydrolysis of subcellular structures and compounds (Fig.11). Furthermore, one of the products of lipid peroxidation, MDA

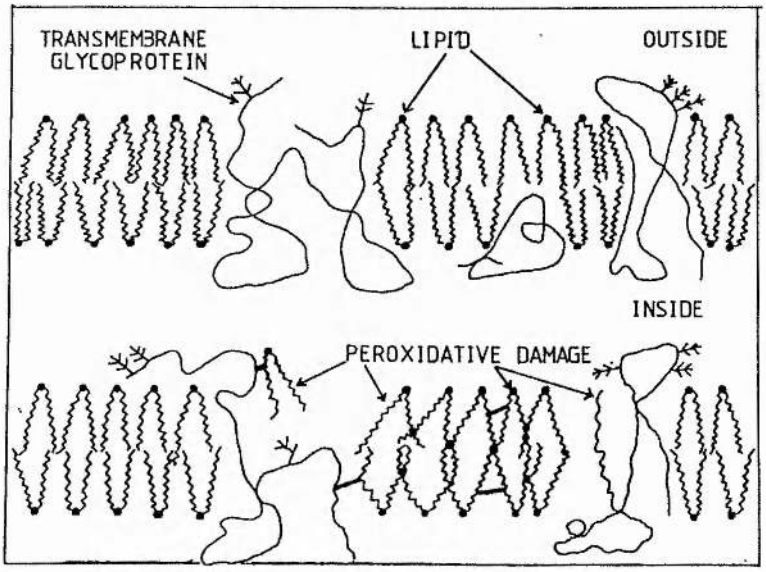


Figure 9. Conceptual depiction of consequences of membrane lipid peroxidation. Upper drawing represents nonperoxidized membrane; lower drawing illustrates peroxidized membrane, with lipid-lipid and lipid-protein cross-links, and loss of membrane protein structural conformation (redrawn from Menzel,1976).

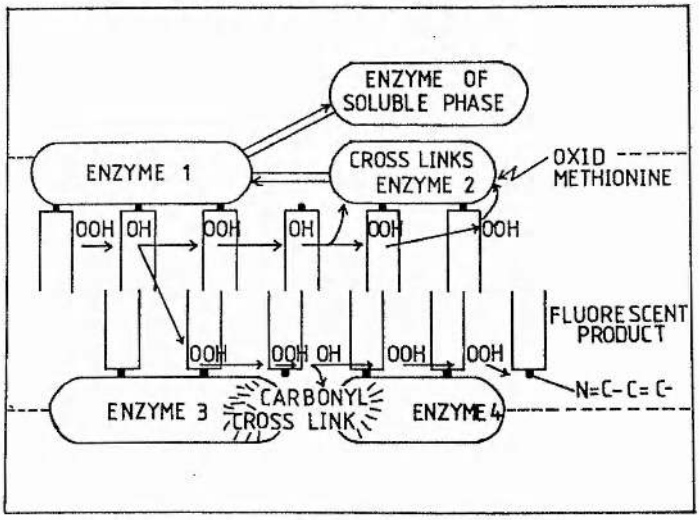


Figure 10. Schematic drawing of a biomembrane in cross section showing propagation of peroxidation of the polyunsaturated fatty acids in the phospholipids. Shown are some of the various types of molecular damage to membrane compounds as a result of the peroxidation reaction (redrawn from Teppel, 1973).

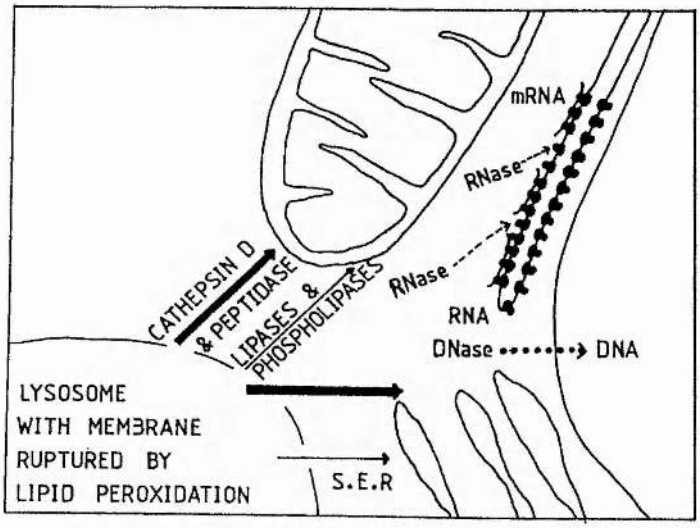


Figure 11. Schematic illustration of the concept of amplification of lipid peroxidation damage through the release of lysosomal hydrolytic enzymes. The release of lysosomal hydrolytic enzymes. The release of enzymes initiate random hydrolysis of subcellular structures and components.

, has been demonstrated to be carcinogenic in mice (Shamberger et al.,1974) as well as to be mutagenic in the Ames system mutagenicity test (Mukai and Goldstein, 1976). All above examples serve to describe the probable wide spectrum of cellular, membrane and molecular pathology that may occur as a consequence of peroxidation of membrane PUFAs. It should be stressed that, although free radical initiations are being produced continuously in cells by both enzymic and non-enzymic reactions, damage does not usually occur owing to the operation of several complex protection mechanisms (see below). Pathological changes result from lipid peroxidation in two kind of situation (i) deficiency in one or more of these defence mechanisms; (ii) abnormally increased production of free radicals which "scramp" the defence mechanisms.

1.5 DEFENCES AGAINST LIPID PEROXIDATION

1.5.1 ENZYMES

1.5.1.1 SUPEROXIDE DISMUTASE

The enzyme superoxide dismutase (SOD) (superoxide oxidoreductase, EC 1.15.1.1) is believed to be present in all O_2 metabolizing cells but lacking in most obligate anaerobes, presumably because its physiological function is to provide a defence against the potentially damaging reactivity of the O_2^{-o} generated by aerobic metabolic reactions (Figs. 12 and 13). Three distinct types of SOD have been described (Fridovich, 1982). They all catalyze the same reaction and do so with comparable efficiency. The iron-containing (FeSOD) and manganese-containing (MnSOD) enzymes are characteristic of prokaryotes and are closely related, as shown by homologies in their amino acid sequences. The enzymes that contain both copper and zinc (CuZnSOD) are characteristic of eukaryotes and appear to have evolved independently, since they have no sequences homologous to those of

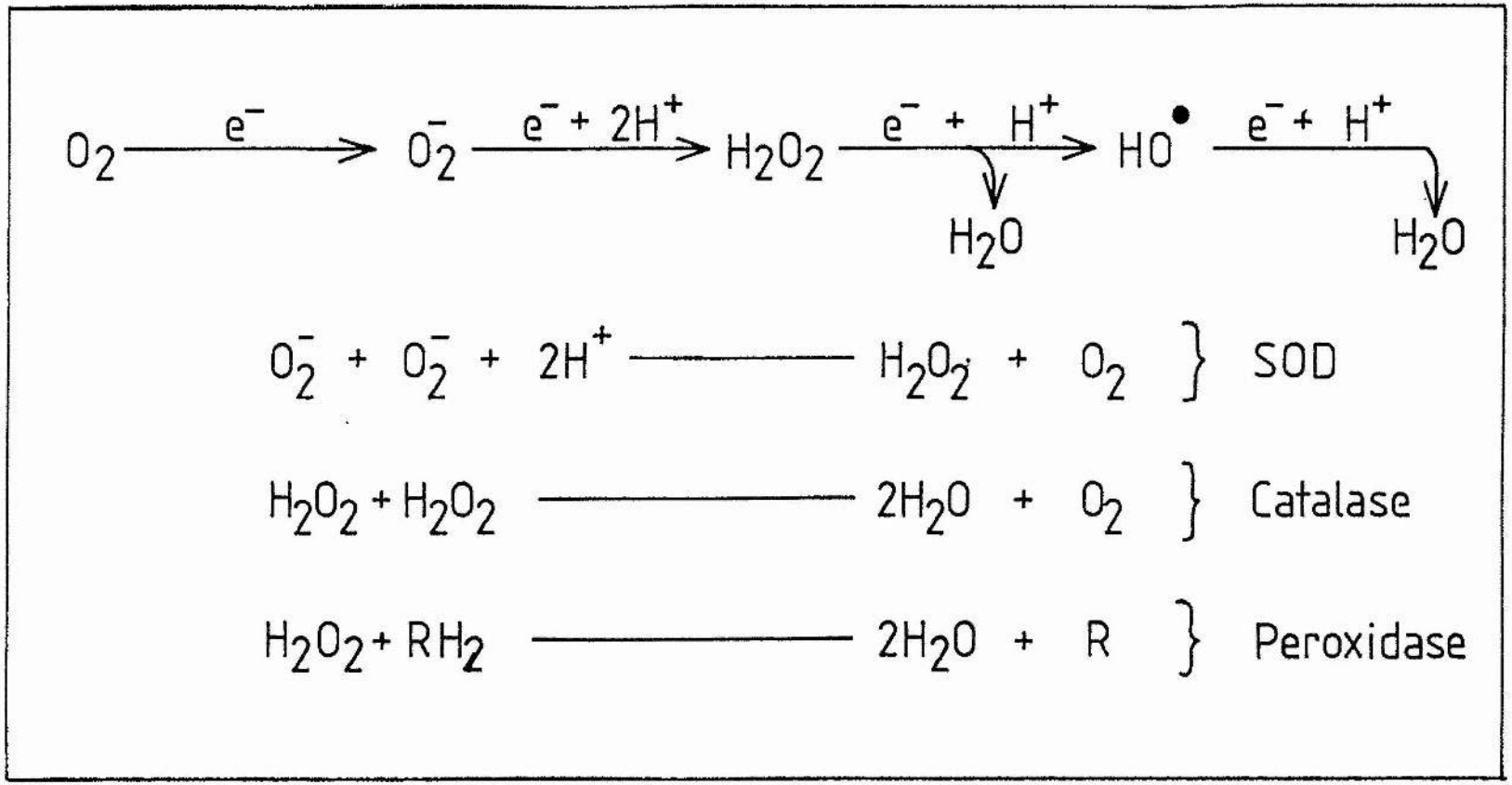


Figure 12. Univalent pathway of oxygen reduction and catalytic scavenging of intermediates (redrawn from Fridovich, 1982).

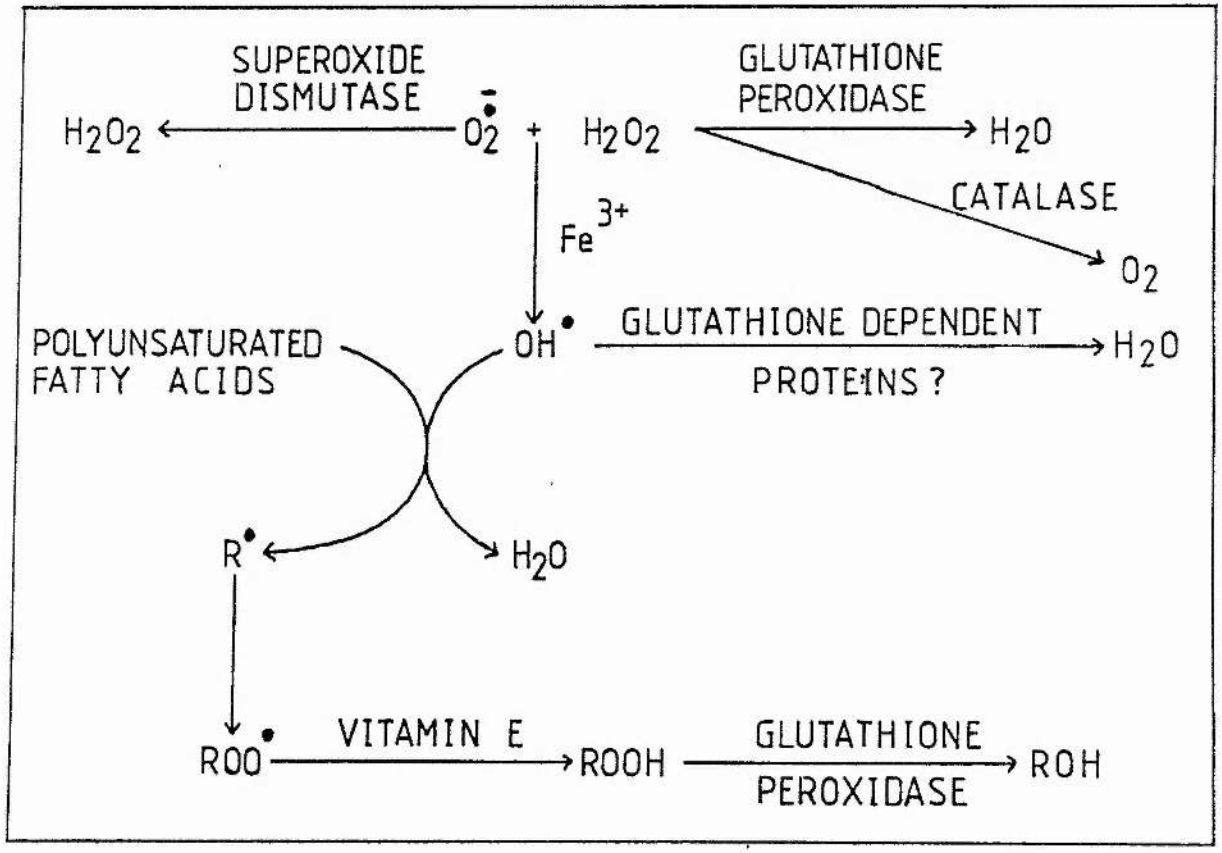


Figure 13. Protective systems in cell against peroxidative reactions. Included in this scheme are superoxide dismutase, catalase, glutathione reductase, glutathione-dependent proteins and vitamin E.

FeSOD and MnSOD (Steinman and Hill, 1973). The biological importance of SOD as a defence mechanism against lipid peroxidation has been demonstrated in a number of elegant studies conducted with bacteria. McCord et al (1971) examined the distribution of SOD among three classes of microorganisms. Aerobes, which utilize O_2 in their metabolism almost exclusively; aerotolerent organisms, which have an anaerobic metabolism even when grown in air; and strict anaerobes which cannot survive in O_2. In all cases, the aerobic organisms contained the highest activity of SOD, followed by intermediate activity in the aerotolerant group. Strict anaerobes examined in this study contained no SOD, which may explain their in ability to tolerate O_2. Further investigations have determined that SOD activity is induced in bacteria (Gregory and Fridovich, 1973) and yeast (Gregory and Fridovich, 1974) in response to lipid peroxidation by O_2 radical. Much of the work done to date regarding all aspects of SOD can be found in an excellent review by Fridovich (1974).

1.5.1.2 CATALASE (CAT)

The enzyme which catalyses the breakdown of H_2O_2 very rapidly is called CAT (Halliwell, 1974). It is almost ineffective at low concentration. This is due to its low affinity (high Km) for H_2O_2. Peroxisomes (single membrane-bound organelles) which contain many of the H_2O_2-generating oxidase enzymes are the most likely candidate for localisation of CAT in most mammalian cells with the exception of RBCs where it is a soluble cytoplasmic enzyme. CAT afforded complete protection against the generation of O_2-derived free radicals in islet cells (by alloxan and dihydroxyfumarate induced diebetes) (Fisher, 1981), in xanthine oxidase promoted lipid peroxidation (Kellogg and Fridovich, 1977), in lung stress following endotoxin administration (bacterial lipopolysaccharide) (Brian, 1981), in neurotoxicity of

nitro compounds (Krinsky et al.,1981), in tissue that was invaded by a large number of metabolically activated inflammatory cells (McCord et al.,1982), in lungs that have been subjected to prolonged exposure to 95%-100% O_2 at a pressure 1atm (Mclennan and Autor, 1982) and in polymorphonuclear leukocytes, macrophages and monocytes which can undergo a respiratory burst on exposure to bacteria and other stimuli (Babior, 1978).

1.5.1.3 THE GLUTATHIONE PEROXIDASE SYSTEM

Glutathione Peroxidase (GSHPx)(glutathione : H_2O_2 oxido-reductase; E.C 1.11.1.9), discovered in 1957 (Mills, 1957) has now come of age in term of its biochemical role. GSHPx has a molecular weight of about 85,000, consists of four g atom of selenium/mol. The three enzymes, GSHPx, GSH Reductase (GSHR) and Glucose-6-Phosphate Dehydrogenase (G6PDhse) (Fig.14) have been referred to as the GSHPx system enzymes (Chow and Tappel, 1972) and are proposed to function as a unit in combating lipid peroxidation as follows: the conversion of toxic lipid hydroperoxides to lipid alcohols (or of H_2O_2 -----> H_2O) by GSHPx is linked to the avtivity of GSHR and G6PDhse, which supply reducing equivalents in the form of reduced GSH and NADPH, respectively (Chow and Tappel, 1972). It has long been known that the enzyme GSHPx can detoxify H_2O_2 and ROOH (Christopherson, 1969). The hydroperoxides accepted as substrates include H_2O_2, ethyl hydroperoxide, thymine hydropreoxide, , hydroperoxides of PUFAs and the corresponding esters, hydroperoxides of steroids and nucleic acids, and prostaglandin G (the primary intermediate of prostaglandin biosynthesis) (Flohe et al.,1976). In vitro GSHPx consistently prevents the oxidative break-down of PUFAs of biomembranes (Flohe et al.,1976). Flohe (1979) has suggested the possibility that this enzymic ability plays a part in the defence

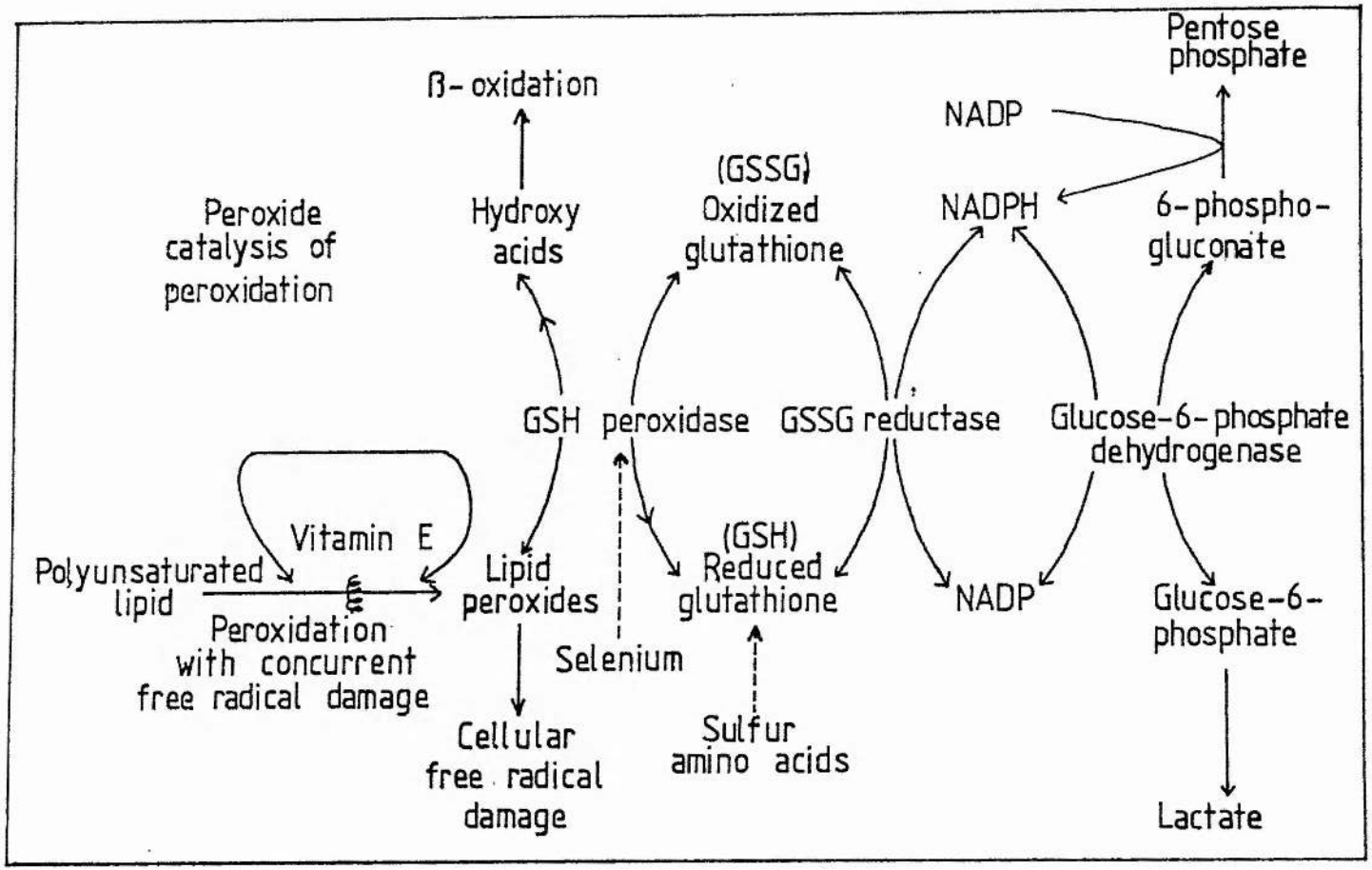


Figure 14. A summary of the reaction mechanisms involved in the action of the glutathione peroxidases-reductase system (redrawn from Chow and Tappel, 1974).

against oxidative damage of organisms living in aerobic conditions (Flohe et al.,1976). It was also observed (Chow et al.,1974) that the activity of GSHR and G6PDhse increased in response to ozone exposure, in addition to the induction of GSHPx. Increased activity of these three enzymes, and SOD, has also been observed in the lungs of rats exposed to 90% O_2 (1 atm) for seven days (Kimball et al.,1976).

Further, McCay et al (1976), working with peroxidizing liver microsomes, could not find the fatty acid alcohol derivatives that Christopherson (1968) suggested should be formed by the action of GSHPx. McCay et al (1976) further showed that if, instead of hydroperoxylinolenic acid (which does form the alcohol), one used a fatty acid hydroperoxide attached to a phospholipid (which is the form of fatty acid in the membrane), the latter did not serve as a substrate for GSHPx. Consequently, without a mechanism for removing the fatty acid, it would be extremely difficult for the fatty acid hydroperoxide (PUFAOOH) and the GSHPx to come together. Thus, their findings suggest that GSHPx may not be able to reduce PUFAOOH in situ esterified to membrane phospholipids.

1.5.1.4 GSH-DEPENDENT PROTEINS

The important role of a GSH-dependent protein(s) (Fig.13) which is not GSHPx as the most crucial intracellular antiperoxidant defence, was first described by McCay et al (1976). Gibson et al (1980) have also reported that GSH-dependent protein(s) (cytosolic-heat labile factor(s))(previously thought to be GSHPx), inhibit lipid peroxidation in both SR and mitochondrial membranes. It is thought that these GSH-dependent protein(s) which inhibit lipid peroxidation in biological membranes do so by preventing radical attack on the PUFAs; i.e at an earlier stage than reduction of already formed ROOH, perhaps by scavenging free radical initiators (Fig.13).

1.5.1.5 CAERULOPLASMIN/TRANSFERRIN: THE FERROXIDASE SYSTEM

Quite early in biological autoxidation reasearch several groups of workers showed that human plasma is a powerful antioxidant (Barber, 1961; Vidlakova et al.,1972). Plasma is generally poor in the enzymes SOD, CAT and GSHPx yet can be subjected to O_2 radicals produced by activated leukocytes, enzymes and autoxidising substances without deleterious effects. Surprisingly, perhaps, the most potent free radical inhibitor both in tissue homogenates and in extracellular fluids was the copper-containing alpha-2-globulin caeruloplasmin (Gutteridge et al., 1982). Copper alone was ineffective, as was the apoprotein (i.e., caeruloplasmin from which the copper atoms had been removed). Caeruloplasmin is not the only antioxidant fraction in plasma: it shares this property with the beta-globulin, transferrin. Caeruloplasmin is not a copper-transporting molecule in the sense in which transferrin is iron-transporting. Caeruloplasmin is not only an oxidase of various organic compounds but also a "ferroxidase". The antioxidant activity (AOA) of transferrin was shown to depend entirely on its ability to bind ferric ions. Since activity could be progressively reversed by saturation with iron (Gutteridge, 1983). The AOA of caeruloplasmin was depend on the intact copper-protein, the apoprotein having no antioxidant activity (Stocks and Gutteridge, 1981). Three possible mechanisms by which caeruloplasmin acts as antioxidant are SOD-like activity, ferroxidase-dependent activity and copper-dependent activity (Gutteridge and Stocks, 1981). The ferroxidase activity of caeruloplasmin (Osaki et al.,1966) and the high affinity binding of the resulting ferric ions to transferrin suggest a co-ordinated role for these two proteins in protection against free radical damage (see Fig.15)(Gutteridge and Stocks, 1981).

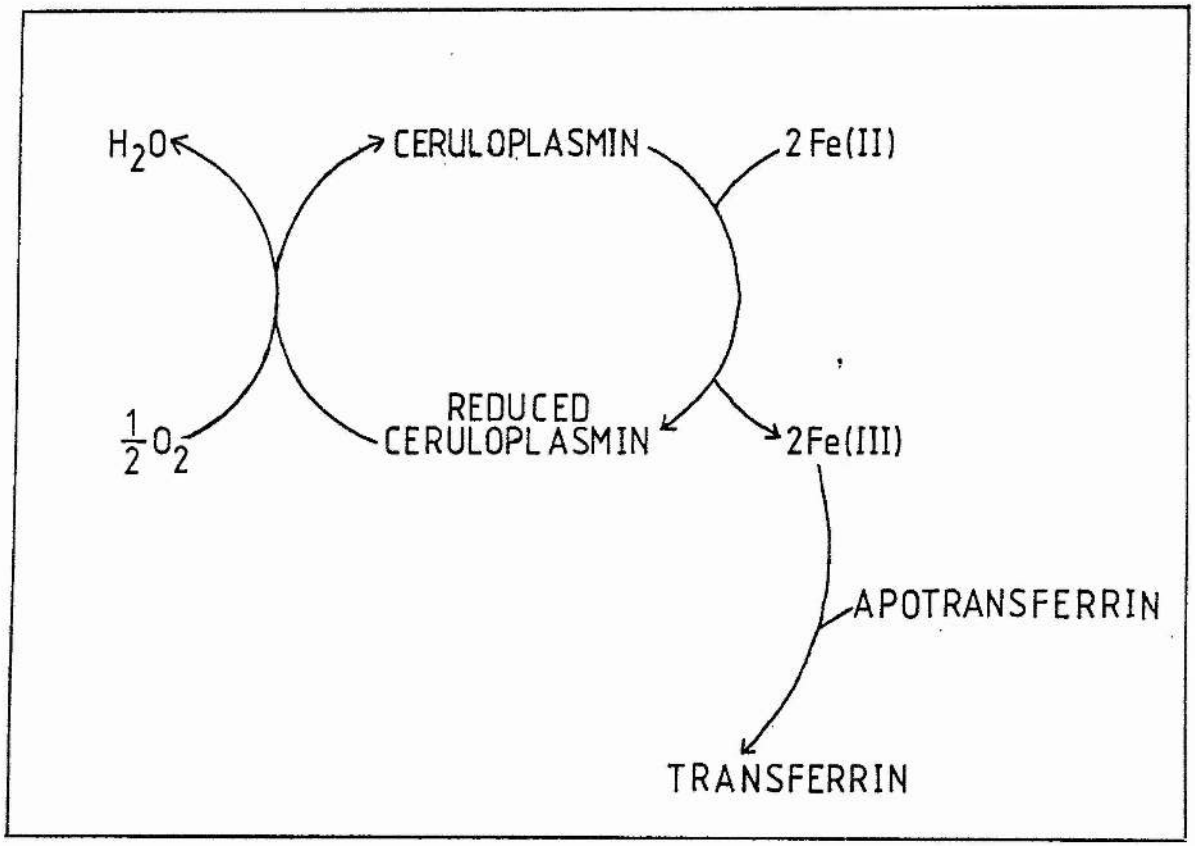


Figure 15. Measurement of ferroxidase activity by monitoring the reaction between apotransferrin and ferric ions to give diferric transferrin.

1.5.2 FREE RADICAL SCAVENGERS

1.5.2.1 VITAMIN C

It has been reported that vitamin C (Fig.16) as well as vitamin E can react with lipid peroxidation products (free radicals) and serve as free radical scavengers or antioxidants (Baehner *et al.*,1982). The important distinction between the two is that vitamin C is water soluble whereas vitamin E is lipid soluble and can therefore react with free radicals within membranes. It was also found that the relative concentration of vitamin C is very important: if the vitamin C concentration is low compared to an oxidising agent, presumably transition metals (iron and copper), then it will be oxidised to the ascorbyl radical which, in turn, will act, as pro-oxidant and will cause lipid peroxidation as mention earlier, in association with iron. However in certain situations the vitamin C radical (ascorbyl radical) can itself be enzymatically reduced to vitamin C by NADH reductase system (Green and O'Brien, 1973)(Fig.17). If, however, the vitamin C concentration is high, then it will acts as an antioxidant. At high vitamin C concentrations no lipid peroxidation can occur, an intermediate concentration is critical for this process. This problem is currently being investigated by Lohmann and his groups (1982). However, Seregi *et al* (1978) suggested that ascorbate may be an antioxidant *in vivo*, while in *in vitro* systems it probably acts to reduce Fe^{3+} to Fe^{2+} thus allowing free-radical production (Jackson *et al.*,1983).

1.5.2.2 VITAMIN E

Vitamin E is a hydrophobic, peroxyl radical trapping chain-breaking antioxidant (Fig.18) found in the lipid fraction of living organisms. It is believed to function largely, if not exclusively, as a lipid antioxidant in *in vivo* (in membranes) (McCay

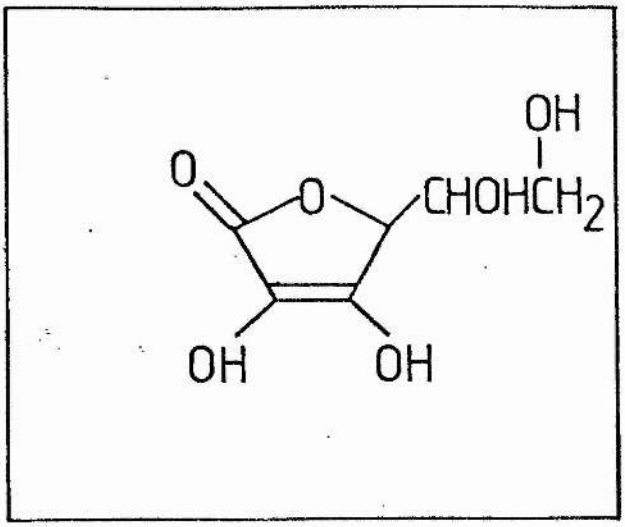


Figure 16. Ascorbic Acid

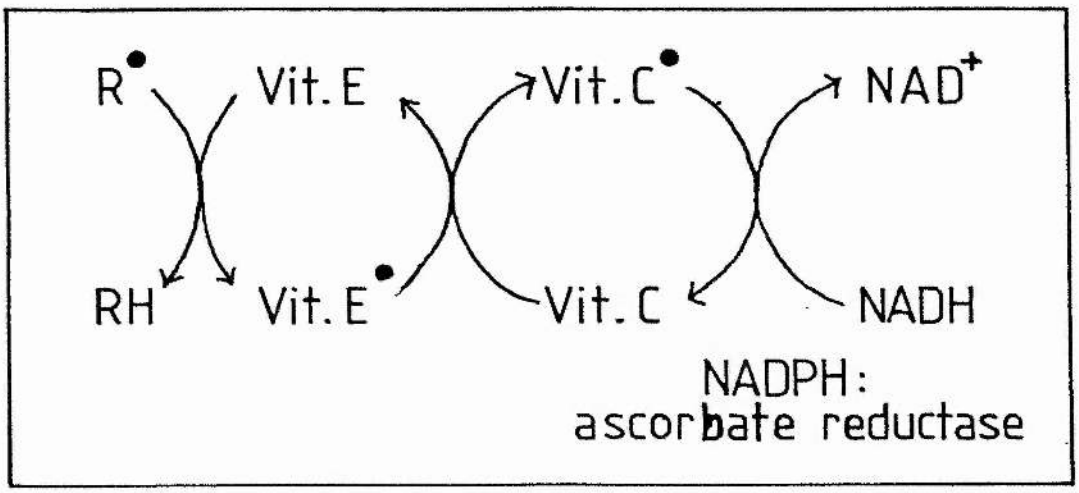


Figure 17. Equation showing redox forms of ascorbate and ascorbyl radical association with vitamin E.

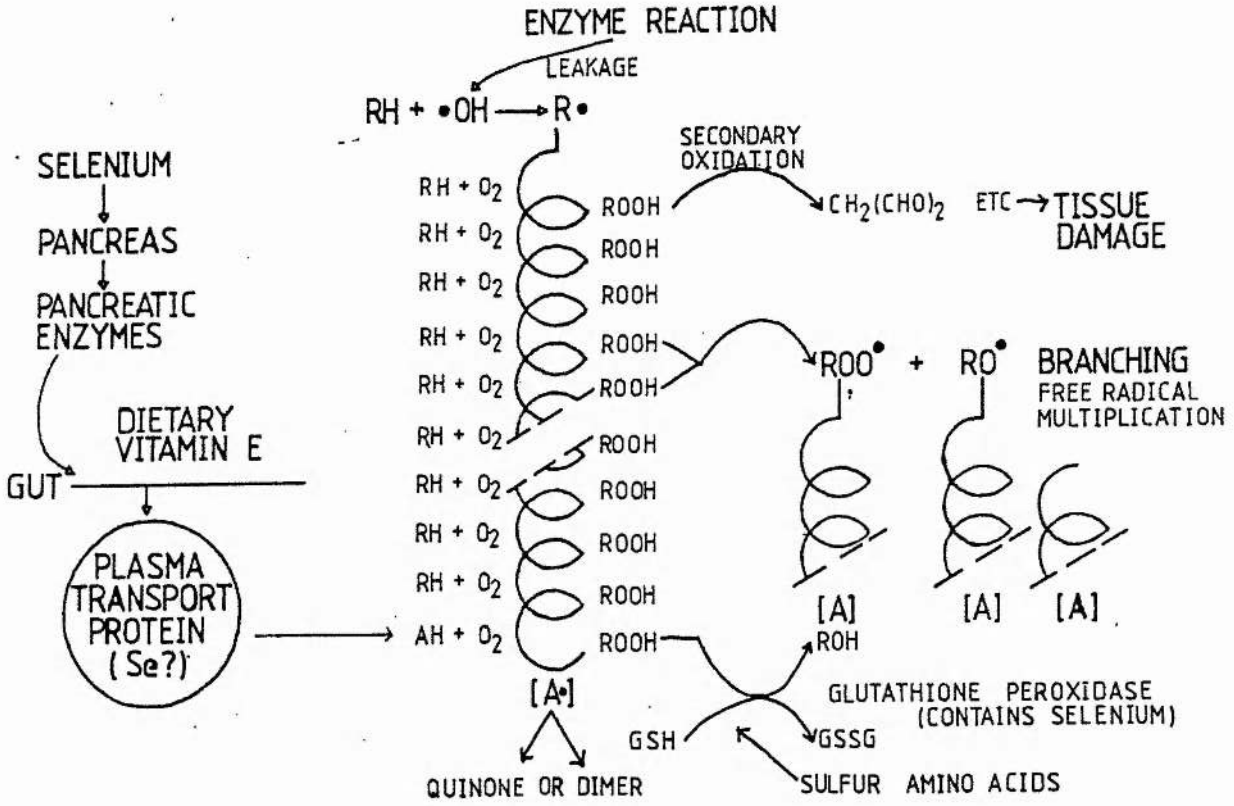
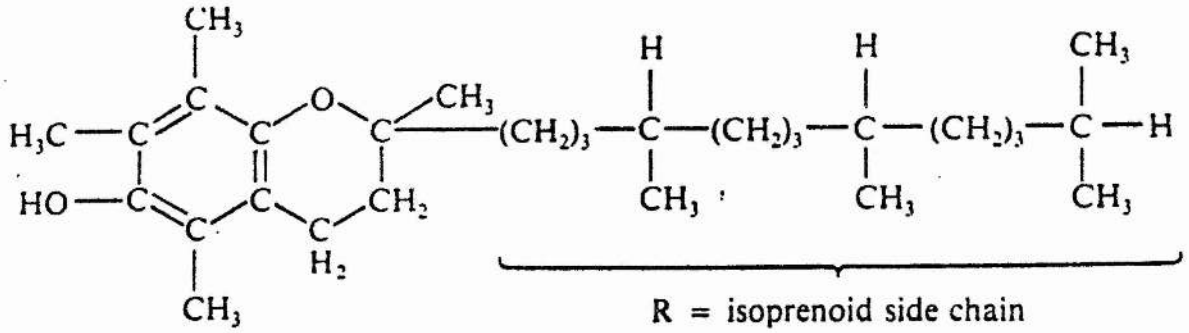


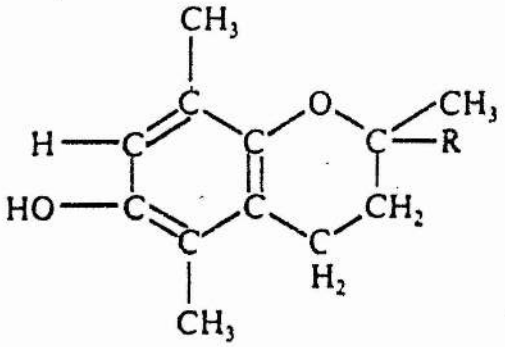
Figure 18. A highly simplified outline of lipid peroxidation in vivo illustrating initiation, chain branching, termination, catabolism of primary products, and secondary reactions leading to tissue damage. (redrawn from Witting, 1975)

and King, 1980) whose role is to protect the lipid material of an organism from the undesireable effects of uncontrolled, spontaneous autoxidation. It may also be an obligatory structural component in membranes containing PUFAs (Diplock and Lucy, 1973) and could act as a catalytic or regulatory agent in metabolism (Schwartz, 1972). Diplock (1983) has studied the interaction between alpha-tocopherol and membrane PUFAs using a tissue culture technique. These findings were interpreted as suggesting that the integrity and maximum functional ability of the membrane depend upon a specific structural interaction of alpha-tocopherol, cholesterol, PUFAs and its derivatives. The role of vitamin E has become clearer as more sophisticated techniques, such as HPLC, have become available for measuring the tocopherols (Fig.19) and for elucidating the pathology of conditions in which an inadequacy of tissue vitamin E content is believed to be involved. The role of vitamin E as an antioxidant has involved areas of research such as carcinogenesis, ageing, toxicology and nutrition. Quintanilha and Packer (1983) have used Bantin-Kingman female rats both *in vivo* and *in vitro* studies. Their results show that a progressive and specific increase in the susceptibility of many subcellular membranes to oxidative damage with increasing levels of vitamin E-deficiency and/or physical stress. In addition, endurance training raised the levels of antioxidant enzymic pathways in both skeletal and cardiac muscle. Several pathological states due to vitamin E-deficiency in animals and humans such as in malabsorption disorders, hematologic disorders, cardiovascular diseases, premature infants (see review Bieri *et al.*,1983), neuropathological diseases in animals and humans (Nelson, 1983), retrolental fibroplasia (Finer *et al.*,1983; Hittner and Kretzer, 1983), nutritional myopathy (McMurray *et al.*,1983), muscle diseases (Jackson *et al.*,1983), menopausal syndrome, infertility, muscular dystrophy and diabetes (Marks, 1962) have been reported.

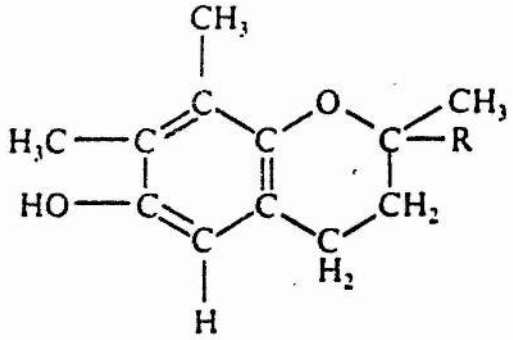
Figure 19. Tocopherol vitamers.



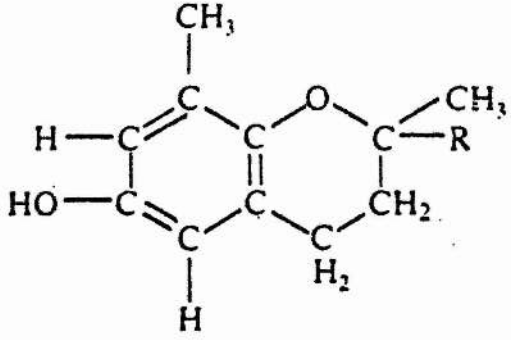
α-tocopherol



ß-tocopherol



γ-tocopherol



δ-tocopherol

1.6 DMD AND LIPID PEROXIDATION: POSSIBLE LINKS

Although several theories have been put forward to explain the pathogenesis of DMD, the exact mechanism has not yet been found and is of great current interest.

The possibility that lipid peroxidation is involved in the aetiology of muscular dystrophy was suggested first in 1974 by Omaye and Tappel, who reported increased MDA in muscle from genetically dystrophic animals.

Kar and Pearson (1979) were the first workers to report increased activity of the enzymes CAT and GSHR and TBA-reactive materials, in human muscle from muscular dystrophy. However these data were pooled from three forms of muscular dystrophy so that their significance with respect to DMD is difficult to assess (Hunter et al.,1981). During the progress of this work, Mechler et al (1984) confirmed the findings (increased TBA-reactive materials) of Kar and Pearson (1979).

Experiments by Burri et al (1980) found a 19% decrease in RBC SOD in DMD patients, suggesting, because of the overlap of dystrophic and control results, that SOD activity changes are secondary to a more primary defect in DMD as not all patients show abnormal SOD activity. GSHPx was normal both in DMD RBC and plasma. In contrast, Hunter et al (1981) reported that SOD, GSHPx and TBA-reactive materials were unaltered from normal in DMD RBC. More recently, Matkovics et al (1982) reported elevated levels of MDA in DMD RBC as well as increased SOD activity. In the most recent paper on this topic Mechler et al (1984) confirmed the findings of Hunter et al (1981) that SOD and TBA-reactive materials in DMD RBC's are not significantly altered from normal. In 1983, Hunter and Amin reported that CAT is unaltered but GSHR is reduced by 20% in DMD RBC.

Recently, Amin and Hunter (1984) reported that SOD, GSHPx, GSHR, and CAT are unaltered in DMD CSFs compared with controls. Despite these contradictory findings, all investigators agreed that lipid peroxidation is still possibly involved in DMD pathogenesis.

A raised level of SM, where no antioxidant was used, is the most common lipid abnormality found in DMD tissue (Rowland, 1980). As several workers point out, this elevated SM may be artifactual and could well result from the prefrential peroxidation, and consequent losses on extraction and chromatography, of the more unsaturated phospholipid classes, PS, PE, and PC. Nevertheless if, as seem likely at least in RBC's, there is no significant difference in fatty acid composition of these phospholipids and if the DMD and control samples were treated in an identical fashion, the fact that SM is found to be "elevated" in DMD samples compared with controls indicates an increased susceptibility of the glycerophospholipids to peroxidative damage in the DMD cells. In addition, a higher activity of endogenous phospholipase A_2 in DMD RBC was also reported (Iyer et al.,1976), and may be evidence of repair of peroxidised phospholipids.

Finally, myopathy in vitamin E-deficient animals (Zalkin and Tappel, 1962) whose muscle has also recently been found to be more susceptible to peroxidative damage (Jackson et al.,1983) is further evidence for possible involvement of lipid peroxidation in myopathy.

1.7 AIMS OF THESIS

From the preceding section it is apparent that there is a body of evidence that membrane lipid peroxidation, if not the primary cause of DMD, may be an important factor in its pathogenesis. Although some previous work has provided evidence for lipid peroxidation in RBCs and muscle, CSFs have not been investigated for this phenomenon. If a generalised membrane defect exists in DMD and increased membrane lipid peroxidation is closely related to the primary defect then one might expect to find evidence for it in CSFs. The first part of the work presented in this thesis was devoted to addressing the following questions:

1. Can increased basal lipid peroxidation be demonstrated in DMD CSFs: (i) in whole monolayer cultures; (ii) in isolated harvested cells; (ii) in isolated membrane-rich fractions?

2. Is lipid peroxidation increased in DMD cells and subfractions when induced by factors such as arachidonic acid, NADPH, Fe/ascorbate?

3. Is the total antioxidant activity of the cytosol fraction from DMD cells abnormal?

Another question of central importance in the issue of the role of lipid peroxidation in DMD is whether evidence of this process in muscle (or other tissues) can be obtained by examining blood plasma/serum in the same way as muscle enzyme leakage is detectable. This might be particularly important in diagnosis of affected individuals and perhaps even of carriers. Plasma was therefore

examined for three indices of lipid peroxidation. The effect of storage of plasma on these parameters was thoroughly investigated.

Alterations in efficiency of defence mechanisms against lipid peroxidation have been invoked by various workers as both the cause and effect of increased tissue lipid peroxidation. Thus decreased activity of glucose-6-phosphate dehydrogenase can lead to increased susceptibility of red cells to peroxidative stress (Chiu et al.,1982), lowered levels of vitamin E result in increased lipid peroxidation and myopathy in animals (Alfin-Slater and Morris, 1963; Jackson et al.,1983) and raised activities of CAT and GSHR are suggested to be responses to the challenge of increased lipid peroxidation in human muscle (Kar an Pearson, 1979; Mechler et al.,1984). It was therefore felt important in this study to assay the important plasma defence systems, namely, vitamin E, caeruloplasmin and transferrin.

2 MATERIAL AND METHODS

2.1 MATERIALS

2.1.1 CHEMICALS and REAGENTS

Agrose (Type III), arachidonic acid, adenosine-5-diphosphate, L-ascorbic acid, ferric chloride, glutathione, niacinamide (nicotinamide; nicotinic acid amide), nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), quinine sulphate, sodium carbonate, sucrose, t-butyl hydroperoxide (TBH), 1,1,3,3-tetraethoxypropane, 2-thiobarbituric acid and tris (hydroxylmethyl)methylamine were purchased from Sigma Chemical company (Poole, Dorset, UK).

Acetic acid glacial, chloroform, Folin and Ciocalteu's phenol reagent, hydrochloric acid, hydrogen peroxide, methanol, phosphoric acid, potassium chloride, sodium azide, sodium hydroxide, sodium lauryl sulphate, sodium tartrate, sulphuric acid, trichloroacetic acid were purchased from BDH Chemical LTD (Poole, England).

Absolute alcohol, butanol (n-butyl alcohol), potassium dihydrogen phosphate, dipotassium hydrogen phosphate and sodium dihydrogen phosphate were purchased from May and Baker Ltd, Dagenham, England. All chemicals and solution used in this investigation were reagent grade, but solvents were dried and redistilled before use (see appropriate section).

Anticaeruloplasmin and antiserum agar gel immunodiffusion plates (LC-Partigen-Caeruloplasmin plate) were purchased from Behring Institute (Behringwerke AG, Marburg-Lahn). Antitransferrin was a gift from the Scotish Antibody Production

Unit (SAPU) (Glasgow and West of Scotland Blood Transfusion service, Law Hospital, Carluke, Lanarkshire, Scotland ML8 5ES). n-Hexane and methanol (HPLC grade) were purchased from Glenrothes chemicals Ltd, (Glenrothes, Scotland). All tissue culture materials were obtained from Gibco (Paisley, Scotland).

2.2 TISSUE CULTURE STUDIES

2.2.1 CELL CULTURE SCHEDULE

2.2.1.1 MEDIA PREPARATION

Serum, L-glutamine and antibiotics were stored at -20°C; remaining ingredients at 4°C till use. Before use they were thawed and incubated at 37°C for 30 minutes. The complete medium was made up as required in a laminar flow cabinet (ENVAIR (UK) Ltd, Haslingden Rossendale, Lancashire, England) (Table: 1).

TABLE (1)

COMPOSITION	VOLUME (ml)	STORAGE TEMP.
Minimum Essential Medium+20mM HEPES	430.0	4°C
Non-essential amino acids (1.0 mM)*	50.0	4°C
Sodium Bicarbonate (75% solution)	5.0	4°C
Penicillin (100 I.U/ml media)	30mg/500ml	4°C
Streptomycin (100 µg/ml media)	50mg/500ml	4°C
Neomycin (10µg/ml media)**	50mg/500ml	4°C
New Born Calf Serum (NBS)	50.0	-20°C
Glutamine 200mM	5.0	-20°C

* The composition of the non-essential amino acids (N.E.A.A) supplement is shown below:

AMINO ACID	CONCENTRATION
L-Alainine	8.9 mg/litre
L-Asparagine	15.0 mg/litre
L-Aspartic Acid	13.3 mg/litre
Glycine	7.5 mg/litre
L-Glutamin Acid	14.7 mg/litre
L-Proline	11.5 mg/litre
L-Serine	10.5 mg/litre

** either neomycin or penicillin + streptomycin were added. 500 ml of medium was made up at any one time. An aliquot (5-10ml) of medium was incubated at 37°C for 3-4 days to check for contamination (sterility test of medium).

2.2.1.2 PHOSPHATE BUFFERED SALINE (PBS) PREPARATION

TABLE (2)

REAGENTS	VOLUME (mg/ml)
Potassium chloride	200.0
Potassium dihydrogen phosphate	200.0
Sodium chloride	8000.0
Di-sodium hydrogen phosphate	1150.0

All reagents were dissolved in water, the pH adjusted to pH 7.4, and made up to volume required. The solution was autoclaved and stored at room temperature.

2.2.1.3 MEDIA CHANGE

Old medium was decanted, the cells washed and fresh medium added at intervals of 3 days.

PROCEDURE:

(1) the medium was decanted into a waste bottle.

(2) the cells were washed by rinsing with 2x5ml of PBS (T75 flask) or 2 x 10 ml PBS (T120 flask).

(3) 10 ml (T75 flask) or 30.0ml (T120 flask) fresh medium was added.

2.2.1.4 SUB-CULTURE

Sub culture was carried out as soon as the monolayer of cells reached confluency. Cells were detached using trypsin and seeded into fresh flasks at a ratio of 1:2 or 1:3.

PROCEDURE:

Spent medium was decanted from the confluent culture. The cell monolayer was briefly washed with 2 x 10ml (T120 flask/bottle) PBS. The cell monolayer was then briefly rinsed with 2.5 ml (T75 flask) or 5.0 ml (T120 flask), or 1.0 ml (T25 flask) of 0.25% trypsin and after which the flask was incubated at 37°C with fresh 0.25% trypsin.

The cells were observed under the light microscope (LM Nikon, Model 41619, Japan), until the cells were seen to have rounded up and begun detachment from the surface of the flask (approximately 1-3 minutes). The cells were completely detached from the substratum by gentle tapping of the flask against a polypropylene "bumb board". The detached cells were mixed with 5.0ml (T75 flask) or 10.0ml (T120 flask) fresh media to neutralize or inactivate the trypsin. The cell aggregates were broken up by repeated gentle pipetting of cell suspension. The cell suspensions were divided into the required number of flasks

(2 or 3 flasks). Each flask was topped up with 10.0 ml (T75 flasks) or 30.0 ml (T120 flasks) fresh medium. Finally, the cells were incubated at 37°C.

2.2.1.5 HARVESTING

This process was used once enough cells had been grown either for storage or immediate use for experiments.

PROCEDURE:

(1) when the cells were confluent, the medium was decanted and discarded.

(2) the cells were washed three times with 5.0 ml PBS (kept at 37°C).

(3) 4.0 ml of 0.25% trypsin solution were added to the flask.

(4) the flask was incubated at 37°C until all cells had been removed (1-3 mins).

(5) 4.0 ml of ice cold PBS was added.

(6) the suspension was transferred into centrifuge tubes (10.0 ml) and centrifuged at 3500xg, for 10 minutes at 4°C.

(7) the PBS was decanted and all pellets were transferred into one centrifuge tube. 10.0 ml of PBS was then added and the tube was centrifuged at 3500xg, for 10 minutes at 4°C.

(8) The combined pellet was washed once more with 10ml PBS as above.

(9) the PBS was decanted and the washed cell pellet

was transferred into small scintillation vials (SV) with not more then 3.0 ml PBS.

The cells were either stored at -70°C for future experiments or used immediately.

2.2.1.6 CRYO-PRESERVATION OF CELLS

The process can be used to cells in a viable state for long periods of time (at least 1-2 years). The cells at confluency were trypsinized as described for sub-culture. Detached cells were mixed with 5.0 ml (T75 flask) or 10.0 ml (T120 flask) fresh media to neutralize or inactivate the trypsin. The suspension was transferred into heat sealable sterile test tubes and centrifuged at 3500xg, for 10 minutes at 4°C. The pellet was resuspended (using a vortex mixer) in 2.0 ml media, supplemented with 10% DMSO and 20% foetal bovine serum (FBS). The cell suspension was transferred into plastic ampoules with screw caps. The ampoules were frozen (at -20°C) for half an hour, then for 24 hours at -60°C and finally stored in liquid nitrogen until use.

2.2.1.7 RECONSTITUTING CELLS

This is the process involved in starting growing cultures from cells which have been frozen in liquid nitrogen for some time.

The ampoules were thawed in water bath at 37°C. The ampoule contents were centrifuged at 3500xg for 7 minutes at 4°C

and the medium decanted (i.e. to remove DMSO). The cell pellets were resuspended (using pipette) in 1-2 ml fresh media. The cell suspension was transferred to a T25 flask (1-2 ampoule contents), 5.0 ml fresh media were added and then FBS added to give a final concentration of 10%. After 24 hours the cells were observed by light microscopy. If the cells were found to have adhered to the flask the medium was changed.

2.2.2 CELL LINES

2.2.2.1 NORMAL CSF LINES

The six normal CSF lines used in this study were obtained as growing monolayers from, Dr. J.A. Witkowski, Jerry Lewis Muscle Research Centre, Department of Paediatrics and Neonatal Medicine, Hammersmith Hospital London, and Dr. G. Priestly, Department of Dermatology, University of Edinburgh. These lines were derived from normal with no family history of genetic disorders. Details are listed in Table (3).

2.2.2.2 DMD CSF LINES

Three cell lines from forearm pinch skin biopsies were derived from three individual subjects confirmed by clinical and biochemical criteria to be DMD sufferers. They were also obtained as monolayer from Dr. J.A. Witkowski, Jerry Lewis Muscle Research Centre, Department of Paediatrics and Neonatal Medicine, Hammersmith Hospital London. Details are listed in Table 3.

2.2.3 CELL CULTURE

Each CSF line (normal and DMD lines) used for study was cultivated under conditions of culture as described in 2.2.1.

Stock cultures of CSFs were maintained as monolayers in 25 cm^2 or 75 cm^2 plastic tissue culture flasks (T25 or T75 flasks, Corning, New York) at 37^{o}C in a STATUS Incubator (Northern Media North Cave, North Humberside, England). Fresh medium was added every 3 days and subculturing carried out every 5-6 days.

TABLE (3)

DETAILS OF CULTURED SKIN FIBROBLAST CELL LINES

DUCHENNE MUSCULAR DYSTROPHY FIBROBLAST CULTURES

CELL LINES	SEX	AGE (yrs)	SOURCE
HAM 1	M	5.2 month	HAMMERSMITH
HAM 4	M	7.9	HAMMERSMITH
HAM 5	M	7.0	HAMMERSMITH

CONTROL CULTURES*

CELL LINES	SEX	AGE (yrs)	SOURCE
HAM 2	M	32	HAMMERSMITH
HSF 26	M	23	EDINBURGH
HSF 6**	M	1	EDINBURGH
HSF 9	M	25	EDINBURGH
HSF 22	M	19	EDINBURGH

* All cultures were from normal individuals with no neurological abnormalities.

** All cell lines were derived from a forearm biopsy site except for HSF 6 which was derived from foreskin.

2.2.4 IN VIVO LIPID PEROXIDATION STUDIES

In vivo lipid peroxidation was studied using an adaptation of the method of Gavino et al (1981).

2.2.4.1 INITIAL CELL CULTURE

Control (HAM 2) and DMD (HAM 5) cell lines, matched for passage number (P10) were grown and subcultured simultaneously to give, for each line, 15 T25 flasks, each seeded with an identical number of cells. These cultures were allowed to grow to confluency in normal growth medium but with FBS instead of NBS. These confluent cultures were then used for incubation in three different media followed by estimation of TBA-reactive materials.

2.2.4.2 EXPERIMENTAL INCUBATION CONDITIONS

2.2.4.2.1 EXPERIMENTAL MEDIA

A. Growth medium (as in TABLE 1) but supplemented with 20% Newborn Bovine Serum (NBS)

B. Arachidonic acid supplemented medium- 5,8,11,14-eicosatetraenoic acid (20:4 ; arachidonic acid) was dissolved in 95% ethanol and diluted 1.6:100 then 1:8 to give a

final 1:500 dilution, with experimental medium, A. (final concentration of 20:4 = 120μM).

C. As B but containing only 95% ethanol.

D. TBH-supplemented medium.
75μl of TBH was added
per 10ml A (final concentration = 300μM).

2.2.4.2.2 CONDITIONS OF INCUBATION

The medium was removed from cultures which had just attained confluency as detailed in 2.2.4.1, the cells washed with PBS and fresh medium, either B, C or D added (t=0). The flasks were incubated at 37°C for varying periods of time, at which duplicate flasks were taken and treated as below for estimation of TBA-reaction materials.

2.2.4.3 ASSAY OF TBA-REACTIVE MATERIALS

2.2.4.3.1 UNWASHED CSFs

After a specified time interval, cells were killed and disrupted by the addition of 2.0ml of 20% trichloroacetic acid to the medium in the flask. Four ml of 0.67% thiobarbituric acid was added, and the medium and cells were incubated for 20 minutes at 97°C. After cooling the medium + cell debris were centrifuged at 12,000xg at 4°C for 10 minutes, to precipitate proteins and cellular debris. The optical density of the supernatant was measured at 532nm (Gavino et al.,1981). A blank containing all

reagents except cells was run in parallel. MDA (sometimes referred to TBA-reactive materials) were calculated from $E^{1\%}_{m}$ for MDA=1.56×10^5 using $O.D_{530}-O.D_{600}$. Absorbance was converted to nmol MDA per culture flask.

2.2.4.3.2 WASHED CSFs

In another set of experiments, washed CSFs were used. The experimental medium was decanted and the cells washed twice with PBS. And then the cells were killed and disrupted by the addition of five ml of 8% trichloroacetic acid to the cells in the flasks. Five ml of 0.54% thiobarbituric acid was added to the flask and TBA-reactive materials assayed as in 2.2.4.3.1.

2.2.4.4 PROTEIN ESTIMATION

The protein content of CSFs was determined according to the method of Lowry (1951).

(i) REAGENTS

(a) 1% cupric sulphate (or cupric sulphate 5-water).

(b) 2% potassium sodium tartrate (w/v).

(c) 2% sodium carbonate in 0.1N sodium hydroxide.

(d) 1N Folin-Ciocalteau's reagent (Stock 2N).

(e) Stock bovine albumin standard solution, 100mg/ml.

(f) Solution A

1% cupric sulphate and 2% sodium tartrate were mixed 1ml + 1ml then made up to 100 ml with 2% sodium carbonate in 0.1N sodium

hydroxide. Solution A is found to be stable for only a few hours.

(g) Solution B

2N Folin-Ciocalteau's Reagent (stock) was diluted 1:1 with distilled water. Solution B was prepared freshly each day.

(ii) PROCEDURE

STANDARD CURVE

The stock albumin solution was diluted to give a protein dilution series ranging from 0-100µg. The stock (100mg/ml) was diluted 1:100 with PBS. Total volume of albumin plus PBS was 0.2 ml.

METHOD

To 0.1 ml sample (in 5.0ml test-tubes) was added 2.5ml solution A. This was incubated for 10 minutes at room temperature and 0.5ml of solution B added. The tubes were mixed and absorbance at 540nm was read against a reagent blank after 30 minutes.

2.2.5 MEMBRANE STUDIES

2.2.5.1 MEMBRANE PREPARATION

Ultrasonication was chosen for cell disintegration as it was fast, efficient and could be used with a small volume (approximately 1.0 ml sample), producing a "concentrated cell suspension". In fact, there are two other methods which can be considered for rupturing the CSF. These are: repeated freezing and thawing and homogenization. Neither of these process were suitable for this particular work owing particularly to the small sample volumes used but also the longer time necessary to achieve complete disintegration.

2.2.5.1.1 METHOD

Frozen samples (stored at -70°C) were defrosted and suspended in a minimum volume (1.0ml) of PBS in a glass scintillation vial. To avoid local heat production by the ultrasonication process, which can induce lipid peroxidation as well as denature membrane protein, samples were always kept on ice. The ultrasonicator probe was inserted just below the liquid surface. Having taken adequate noise protection precautions, the sample was sonicated at an amplitude of 8-9 microns peak for 1 minute, with a break for 30 seconds to allow adequate cooling. This was repeated to give each sample a total sonication time of 15-30 minutes. The sonicates were then spun as follow: The sonicated CSFs were transferred into 10x10ml polypropylene centrifuge tube (Model 34411-117 MSE) and were centrifuged at 124,000xg in a 10x10ml Titanium angle rotor in the Prepspin 50 refrigerated Ultracentrifuge (GK-100, Measuring and Scientific Equipment Ltd, Manor Royal, Crawley, Sussex, England) for 60 minutes at 4°C. The supernatant (cytoplasmic fraction) was removed and stored at -70°C for antioxidant studies (see section 2.2.7). The pellet was washed in tris-HCl (10mM) pH 7.4 with 0.15M potassium chloride and re-centrifuged at 124,000xg as above. The pellet was washed twice and the washings removed and discarded. The pellet (membrane fraction) was resuspended in tris-HCl (10mM) pH 7.4 + 0.15M potassium chloride and the total volume of membrane suspension measured. Protein was determined (in duplicate) as below. The rest of the membrane suspension was stored at -70°C, prior to further experiments.

Notes:

(1) All procedures were carried out at 0-4°C.

(2) Ice-buckets were used for all transfers.

(3) Solutions and centrifuge rotors and tubes were pre-cooled prior to use.

(4) Resuspension may necessitate resonication.

2.2.5.1.2 PROTEIN ESTIMATION

The protein content of CSF membrane suspensions was estimated according to the method of Lowry (1951). A 50µl sample was taken, the total volume of the membrane suspension in tris-HCl (10mM) pH 7.4 with 0.15M potassium chloride already having been estimated, this was digested with the same volume of 0.2M sodium hydroxide for 5 minutes at room temperature. 30µl aliquots were then used to carry out the estimation as in 2.2.4.4. Bovine serum albumin, 0-100µg in 0.1ml was used as a standard.

2.2.5.1.3 ENZYMIC LIPID PEROXIDATION

Enzymic lipid peroxidation is an NADPH-dependent process (also requiring of ADP-chelated iron) and was determined according to the method of Koster and Slee (1980).

INCUBATION MEDIUM

20mM tris-HCl pH 7.0

2.0mM adenosine-5-diphosphate

0.12mM ferric chloride

20mM nicotinamide

27mM potassium chloride

4.0mM NADPH

Plus 1-3.25mg protein/ml incubation medium

Temperature = 37°C

Time course = 0-180 minutes.

METHOD:

CSF membrane suspension in 10mM tris-HCl, pH 7.4 + 0.15M potassium chloride was added (1-3.25mg protein/ml incubation medium) to incubation medium (as above). Total volume of incubation is 6.0ml. Samples for 0 time were taken (in duplicate) immediately. The rest of the sample was incubated at 37°C. Samples of 0.5ml and 0.2ml were withdrawn at 60, 120 and 180 minutes to measure MDA and determine FPs, respectively.

2.2.5.1.4 LIPID EXTRACTION + FP DETERMINATION

Lipid extraction was carried out by a method modified method from Koster and Slee (1980). All solvents were dried and redistilled prior to use. Butylated hydroxy toluene (BHT) was added to a concentration of 5.0mg %. All glass-ware was soaked overnight in Decon 90 before use.

METHOD

0.2ml CSF membrane suspension (+ incubation medium) were transferred into a 30ml Q and Q centrifuge tube. The suspension was extracted with 2.0ml chloroform/methanol (2:1, v/v). The mixture was shaken intermittently for 1-2 hours. Then the tube was centrifuged at 12,000xg for 10 minutes at 4°C. The supernatant (Supernatant 1) was removed and transferred into another Q and Q centrifuge tube. To the pellet was added 2.0ml chloroform/methanol (2:1, v/v). The tube was again shaken

intermittently over 1-2 hours and then centrifuged at 12,000xg for 10 minutes at 4°C as above. The supernatant (supernatant 2) was carefully removed. The pellet was discarded. The first and second supernatants were pooled and diluted with 0.625 ml chloroform and 0.625 ml 0.05N potassium chloride. The tube was centrifuged and the upper layer (methanol/water/potassium chloride) was discarded while the lower layer (chloroform-rich layer) was removed into a 25ml round bottomed flask. The solution was rotary evaporated to a small volume. The sample was then transferred in chloroform/methanol (1:1, v/v) to a small vial and dried on a heating block (Dri-Block DB-3 Techne) at 30°C under a stream of nitrogen. The sample was then taken up in exactly 2.0ml of chloroform/methanol (1:1, v/v) and used immediately or stored at -60°C prior to FP estimation.

1.0ml of chloroform/methanol extract was withdrawn and 0.1ml methanol added to clarify the sample. Fluorescence spectra were measured in an Aminco-Bowman spectrophotofluoremeter (American Instrument Company, Division of Travenol Laboratories, Inc, 8030 Georgia Avenue, Silver Spring, Maryland 20910), with quinine sulphate in 0.05M sulphuric acid as standard. Fluorescence emmission/absorption was expressed as relative fluorescence units per mg protein.

2.2.5.1.5 MDA ESTIMATION

To the 0.5 ml sample (see section 2.2.4.3) 0.5 ml 35% trichloroacetic acid were added. After 10 minutes incubation and intermittent shaking at room temperature, 0.5ml 50mM Tris-HCl, pH 7.4 were added. The suspension was centrifuged, at 12,000xg for

10 minutes. After centrifugation, 1.0ml of 0.75% thiobarbituric acid [75mM of TBA in sodium hydroxide (50mM)] were added. A glass bead (or marble) was put on the test tube as a cap and the mixture was heated for 30 minutes in a boiling water bath. After cooling, the solution was diluted with 2.0ml 35% trichloracetic acid and the optical density of the supernatant was determined at 532nm. In these experiments, appropriate blanks are necessary. When the colour intensity was too high, the optical density was measured after the solution was diluted with 35% trichloroacetic acid. MDA content was calculated from $E^{1\%}_{m}$ for MDA=1.56 x 10^5 using $O.D_{530}-O.D_{600}$ and a scale expansion facility to give extra sensitivity. The values were converted to nmol MDA per mg membrane protein.

2.2.6 IN VITRO PEROXIDATION OF WHOLE CSF HOMOGENATES

2.2.6.1 SONICATION AND HOMOGENATE PREPARATION

Frozen samples (stored at $-70^{\circ}C$) were defrosted and suspended in a minimum volume (2.0ml) of PBS. Samples were kept on ice at all times. The cell suspensions were further disrupted by sonication using a ultrasonicator probe in MSE Ultrasonicator. The cell suspensions on the ice were subjected to three cycles of sonication (30 seconds on/30 second off per cycle) with a power setting of 8. This was repeated to give each sample a total sonication time of 30 minutes. The sonicated homogenates were used for determination of enzymic and non-enzymic *in vitro* induced lipid peroxidation as well as protein determination.

2.2.6.2 PROTEIN DETERMINATION

The protein content of CSF sonicated homogenates was estimated according to the method of Lowry (1951). A 0.1ml sample was added to 5.0ml solution A (see section 2.2.4.4). This was incubated for 10 minutes at room temperature and 0.5ml of solution B (see section 2.2.4.4) were added. The tube contents were mixed and absorbance at 540nm was read against a reagent blank after 30 minutes.

2.2.6.3 ENZYMIC IN VITRO LIPID PEROXIDATION

Enzymic *in vitro* lipid peroxidation was determined in whole cell homogenates (see section 2.2.6.1). Sonicated cell homogenates (1-3.25mg protein per ml incubation medium) was added to a test tube and brought to a final volume of 6.0 ml with incubation medium (as in 2.2.5.1.3). The tubes were incubated with intermittent shaking for 180 minutes in a 37^{o}C water bath, samples for 0 time were taken immediately after adding NADPH and further samples were taken at 60, 120 and 180 minutes. Separate 0.5ml samples (in duplicate) were withdrawn at each time to measure thiobarbituric acid-reactant materials (MDA), FPs, (as described earlier) and also CDs.

2.2.6.4 NON-ENZYMIC IN VITRO LIPID PEROXIDATION

Non-enzymic *in vitro* lipid peroxidation was induced and measured in whole cell homogenates. This is ascorbate-dependent lipid peroxidation (also requiring ADP-chelated iron (III) salt) and was carried out according to the modified method of Koster and Slee (1980).

INCUBATION MEDIUM

4mM adenosine-5-diphosphate

12μM ferric chloride

0.66mM ascorbic acid

10mM gluthatione (GSH)

Homogenate: 1.0mg protein per ml incubation medium

Time course = 0, 60, 120, and 180 minutes

Temperature = 37°C

METHODS

CSF homogenate (in 10mM tris-HCl pH 7.4 buffer + 0.15M potassium chloride) was added (1.0mg protein per ml incubation medium) to incubation medium (as above). Samples for 0 time were taken immediately after adding ascorbate/Fe^{3+} to medium. The rest of the sample was incubated at 37°C. Duplicate 0.5ml samples in two portion were withdrawn at 60,120 and 180 minutes to measure thiobarbituric acid-reactant materials (MDA), FPs activity and CDs.

2.2.6.5 LIPID EXTRACTION AND FP MEASUREMENT

This procedure was carried out as in 2.2.5.1.4 except that 0.5ml samples (in duplicate) were taken from the incubations and the volumes of solvents used for extraction increased in proportion.

2.2.6.6 CD ESTIMATION

CD content of chloroform/methanol (2:1, v/v) extracts of whole CSF homogenates were analysed by measuring absorbance at 233nm as an indicator of lipid peroxidation (Dodge and Phillips, 1966). All measurements were performed against a solvent blank. CDs were expressed as relative absorbance (233nm) per mg whole CSF protein.

2.2.6.7 MDA ESTIMATION

MDA activity in incubated sonicate CSF homogenates were estimated as described in section 2.2.5.1.5 above.

2.2.7 ANTIOXIDANT STUDIES

The activities of the antioxidant enzymes, CAT, SOD, GSHPx, GSHR recognised as being physiologically important, have been investigated in DMD muscle (Kar and Pearson, 1979; Mechler et al.,1984) and DMD RBC (Buri et al.,1980; Hunter et al.,1981; Matkovics et al.,1982 and Hunter and Amin, 1983). Some changes have been reported but the results, especially with RBC, are somewhat contradictory. In our laboratory we have also found that the activities of these enzymes are not different from normal in DMD CSFs (Hunter, personal communication).

Nevertheless in view of the possible existence of other uncharacterised antioxidants it was felt desirable to test the "total antioxidant activity" of the soluble fraction from DMD CSFs. An in vitro system based on autoxidising rat liver particulate fraction was set up to assay this activity as this system has been well characterised and sufficient material was

not available to use the same fraction from CSFs.

2.2.7.1 SUPERNATANT FRACTION

The supernatant fraction from sonicated CSFs (2.2.5.1) was used for these experiments. The protein content of the supernatant was first estimated by the method of Lowry (1951) as in 2.2.4.4.

2.2.7.2 PARTICULATE PREPARATION

The particulate fraction from rat liver was isolated according to the method modified after Koster and Slee (1980).

REAGENTS

(1) Sucrose, 0.25M neutralized to pH 7.0 with 0.15mM potassium hydroxide.

(2) Homogenization medium: 0.25M sucrose + 10mM tris-HCl, pH 7.4.

FRESH LIVER

Male rats obtained from the Animal House, University of St.Andrews, Scotland were used for all experiments. The animals were allowed a normal food and water supply throught the experiments.

The rats were killed instantly without anaesthesia. The livers are rapidly excised, immersed in ice-cold 0.25M sucrose, and cut into several large pieces. The pieces of liver were

blotted on absorbent paper, weighed and placed into 5 volumes of homogenization medium. The liver pieces were further minced using a pair of scissors and then subject to homogenization (as below).

HOMOGENIZATION PROCEDURE

The tissue in homogenization medium was transferred to a 50ml glass homogenizer (cooled prior to use) and homogenized using a Potter Elvehjem type tissue grinder (A.H. Thomas, Philadelphia, Pennsylvania). The Teflon pestle was driven mechanically at approximately 3500xg. The time of homogenization for each up-and-down strokes (3-5 strokes) was about 5 seconds. The homogenate was centrifuged at 600g at 4°C for 10 minutes. The pellet (cell debris and nuclei) were discarded. The supernatant was transferred into 10x10ml polypropylene centrifuge tubes. The tubes were centrifuged at 124,000xg in 10x10ml Titanium angle rotor in an MSE Automatic Superspeed 65 Ultracentrifuge for 60 minutes at 4°C. The supernatant (cytoplasmic fraction) was removed and stored at -70°C for future analysis. The pellet was again resuspended in 10mM tris-HCl, pH 7.4 as above except with 0.15M potassium chloride (instead of 0.25M sucrose). The mixture was centrifuged at 124,000xg for 60 minutes at 4°C as above. This was repeated twice. The supernatants were removed by suction and discarded. The pellet (particulate fraction) was resuspended in a small volume of 10mM tris-HCl pH 7.4 + 0.15M potassium chloride. The total volume was measured and the protein content estimated as in 2.2.4.4. The suspension was stored at -70°C.

Note:

(1) All procedures were carried out at 0-4°C, in cold room, or refrigerated centrifuge.

(2) Ice bucket was used for transfers

(3) All solutions, homogeniser vessel centrifuge tubes (10x10ml) and rotor, precooled.

(4) Resuspension may necessitate resonication.

2.2.7.3 CSF SUPERNATANT STUDIES - ANTIOXIDANT PROPERTIES

Prior to experiments with CSF supernatant fraction, control experiments were carried out without addition of supernatant, but otherwise under the conditions described below for enzymic and non-enzymic lipid peroxidation to determine the optimum time interval for incubation.

2.2.7.3.1 ENZYMIC LIPID PEROXIDATION

The incubation medium contained approximately 1-3.25mg particulate protein per ml and 3.38mg supernatant protein per mg of particulate protein per ml incubation medium (see 2.2.5.1.3). To initiate the enzymic lipid peroxidation reaction, 4mM NADPH was added to the system. 0.5 ml samples for 0 time were taken immediately after adding NADPH to the system. The rest of the samples were incubated for 60 minutes. Then, 0.5ml samples were withdarwn for lipid peroxidation product estimation.

2.2.7.3.2 NON-ENZYMIC LIPID PEROXIDATION

The incubation system contained approximately 1mg of particulate protein and 3.38mg supernatant protein per ml incubation medium (as in 2.2.6.4). To initiate the non-enzymic lipid peroxidation, 0.66mM ascorbic acid were added to the system. 0.5ml samples for 0 time were taken immediately after adding ascorbic acid to the system. The rest of the suspension was incubated for 60 minutes at 37^{o}C. Then 0.5ml samples were withdrawn for lipid peroxidation product determination.

2.2.7.3.3 LIPID EXTRACTION

Lipid extraction was carried out according to the method described in section 2.2.5.1.4.

2.2.7.3.4 MDA ESTIMATION

MDA was estimated according to the method described in section 2.2.5.1.5.

2.2.7.3.5 CD ESTIMATION

CDs were assayed according to the method described in section 2.2.6.6.

2.2.7.3.6 FP ESTIMATION

FPs were estimated according to the method described in section 2.2.5.1.4.

2.3 BLOOD PLASMA STUDIES

2.3.1 BLOOD SAMPLING AND WASHING PROCEDURES

2.3.1.1 NORMAL BLOOD PLASMA

A total of 53 normal human blood plasma samples were used in this study, obtained from 10ml whole blood was freshly drawn by venepuncture into lithium-heparin tubes from healthy controls. Donor were staff or out-patients from Ninewell Hospital, Departments of Paediatrics and Biochemical Medicine, Dundee as well as staff and students from Department of Biochemistry and Microbiology, University of St.Andrews. Most normal donors were completely healthy. Young males (9-16 years)

were patients attending a paediatrics out-patient clinic but who were not suffering from muscular or neuromuscular disease. Samples are listed in Table 4.

2.3.1.2 DMD BLOOD PLASMA

A total of 27 DMD plasma samples were used in this study, obtained from 10ml of whole blood as above. The 27 individual subjects were confirmed by clinical and biochemical criteria to be DMD sufferers. They were also obtained from Ninewell Hospital as well as from Department of Medicine, University College of London. They are listed in Table (5).

2.3.1.3 BLOOD WASHING PROCEDURE

10ml of blood (see section 2.3.1.1 and 2.3.1.2) was centrifuged at 1075xg for 10 minutes. The plasma was aspirated and stored at -20^{o}C for future experiment. The buffy coat and RBC were discarded.

TABLE (4)

BLOOD PLASMA INFORMATION

NORMAL CONTROL BLOOD PLASMA[a]

PATIENT NAME	DATE OF SAMPLING	SEX
PN	25.06.80	M
AS	25.06.80	M
CV	25.06.80	M
JG	25.06.80	M
NP	25.06.80	M
PdV	28.06.80	M
MSB	23.07.80	M
IMcL	23.07.80	M
DJ	28.07.80	M
ID	31.07.80	M
DK	31.07.80	M
MISH	09.05.81	M
MSL	09.05.81	F
DK	23.05.81	M
JBM	23.05.81	M
AH	31.07.82	M
MISH	10.10.82	M
SA	10.10.82	F
JB M	10.10.82	M
DK	12.12.82	M
MSL	12.12.82	M

a = age between 18 and 35 years.

TABLE (4)

(CONTINUED)

NORMAL CONTROL BLOOD PLASMA[b]

PATIENT NAME	DATE OF SAMPLING	SEX
MSL	09.05.84	F
MB	17.05.83	M
DK	23.05.83	M
AI	23.05.83	M
PR	23.05.83	M
AT	23.05.83	M
MISH	25.05.83	M
JBM	25.05.83	M
DS	25.05.83	M
DL	25.05.83	M
AG	25.05.83	M
LC	25.05.83	M
JQ	30.05.83	M
EJ	13.06.83	M
ML 1	24.08.83	M
ML 2	24.08.83	M
JDB	24.08.83	M
BCN	18.11.83	M
AL	18.11.83	M
JH	18.11.83	M
J M	18.11.83	M
PN	18.11.83	M

b = age between 20 and 35 years.

TABLE (4)

CONTINUED

NORMAL CONTROL PLASMA (CHILDREN SAMPLES)[c]

PATIENT NAME	DATE OF SAMPLING	SEX
PA	24.1.84	M
A	24.1.84	M
LH	24.1.84	M
MG	26.1.84	M
HT	16.2.84	M
FF	16.2.84	M
GR	16.2.84	M
AE	20.2.84	F
EP	19.4.84	M
GP	19.4.84	M

c = age between 4 and 16 years.

TABLE (5)

DUCHENNE MUSCULAR DYSTROPHY BLOOD PLASMA[d]

PATIENT NAME	DATE OF SAMPLING	SEX
TB	17.07.80	M
IB	17.07.80	M
DB	17.07.80	M
RT	17.07.80	M
D 1	17.07.80	M
D 2	17.07.80	M
D 3	17.07.80	M
ND	12.11.80	M
RT	17.07.81	M
BJ	28.08.81	M
DD	12.11.81	M
D 4	05.04.82	M
Md	09.06.82	M
D 5	22.06.82	M
D 6	29.06.82	M
DS	03.08.82	M
DG	03.08.82	M
D 7	12.11.82	M

d = age between 4 and 12 years

TABLE (5)

(CONTINUED)

DUCHENNE MUSCULAR DYSTROPHY BLOOD PLASMA[e]

PATIENT NAME	DATE OF SAMPLING	SEX
DC	31.05.83	M
SO	31.05.83	M
JT	02.06.83	M
DT	02.06.83	M
NRJ	02.06.83	M
MF	02.06.83	M
JM	17.01.84	M
DR	22.01.84	M
PM	16.02.84	M

e = age between 4 and 16 years.

2.3.2 FRESH PLASMA STUDIES

2.3.2.1 LIPID EXTRACTION

0.5ml fresh human blood plasma was transferred to a Q and Q centrifuge tube (in duplicate) and extracted with 5.0 ml chloroform/methanol (2:1, v/v). The tubes were shaken intermittently for 1-2hours at room temperature. Then the tubes were centrifuged at 12,000xg for 10 minutes. The upper layer and precipitated protein at the interface were discarded. Then 2.0ml chloroform and 2.0ml of 0.05N potassium chloride were added into the tubes. The tube contents were mixed thoroughly on a vortex mixer at high speed and then centrifuged at 12,000xg as above. The upper layer (methanol/water/potassium chloride) were removed and discarded. The lower layer (chloroform-rich layer) was removed and transferred into a 10ml test tube and dried under nitrogen. The dried residues were then dissolved in 5.0ml chloroform. The lipid aliquot was stored at -20^{o}C for lipid peroxidation products estimation.

2.3.2.2 MDA ESTIMATION

2.3.2.2.1 METHOD 1

This method was based on that of Koster and Slee (1980). To 0.5ml sample, 0.5ml 35% trichloroacetic acid and 0.5ml 50mM tris-HCl, pH 7.4 were added. After centrifugation and addition of 1.0ml 0.75% thiobarbituric acid, the mixture was boiled for 15 minutes. After cooling, 1.0ml 70% trichloroacetic acid was added and the absorbance read at 532nm.

2.3.2.2.2 METHOD 2

This method was based on that of Satoh (1978). To the 0.5ml plasma, 2.5ml of 20% trichloroacetic acid was added and the tube was left to stand for 10 minutes at room temperature. After centrifugation at 12,000xg for 10 minutes, the supernatant was decanted and the precipitate was washed once with 0.05M sulfuric acid. Then 2.5ml of 0.05M sulfuric acid and 3.0ml of 0.2% thiobarbituric acid in 2M sodium sulfate were added to this precipitate and the coupling of lipid peroxide with thiobarbituric acid was carried out by heating in a boiling water bath for 30 minutes. After cooling in cold water, the resulting chromogen was extracted with 4.0ml of n-butyl alcohol by vigorous shaking. Separation of the organic phase was facilitated by centrifugation at 12,000xg for 10 minutes and its absorbance determined at 530nm.

2.3.2.2.3 METHOD 3

This method was based on that of Slater (1971). 2.5ml of 20% trichloroacetic acid and 1.0ml of 0.67% thiobarbituric acid were added to 0.5ml of serum, and the mixture heated in a boiling water bath for 30 minutes. The resulting chromogen was extracted with 4.0ml n-butyl alcohol and the absorbance of the organic phase determined at 530nm.

2.3.2.2.4 MODIFIED METHOD

This method was modified to solve some problems which arose from each of the above methods. Only method 3 developed a

stable TBA-MDA colour. The chromophore was unstable with method 1 and was not developed at all with method 2. The reason for the failure of development with method 2 may be due to the low trichloroacetic acid concentration (0.2mg/ml) which may not be sufficient to precipitate the total plasma protein. The affinity of TBA to react with MDA during boiling ($100^{o}C$) may also be reduced since 0.02mg/ml may not be adequate to produce a high intensity of TBA-MDA colour. A large broad peak at 450nm which interferes with the MDA peak at 532nm made method 3 unsuitable for MDA estimation (especially plasma). This interference was suggested by Satoh (1978) to be due to the reaction of sialic acid with TBA. This may explain the unstable colour developed with method 1 and prompted Satoh (Method 2) to use sodium sulfate (2M) which he claimed helped to reduce the interfering peak at 450nm. A modified method (below) was therefore developed to overcome these difficulties. To 0.5ml plasma 0.5ml 35% trichloroacetic acid was added and the tube left to stand for 10 minutes at room temperature. Then 0.5ml 50mM tris-HCl, pH 7.4 were added. After centrifugation at 12,000xg for 10 minutes, the supernatant was decanted and the precipitate was washed once with 0.05M sulfuric acid. Then 1.0ml 0.75% thiobarbituric acid in 2M sodium sulfate were added to this precipitate and mixed thoroughly. The coupling of lipid peroxide with thiobarbituric acid was carried out by heating in a boiling water bath for 45 minutes. After cooling at room temperature, the resulting chromogen was extracted with 4.0ml n-butyl alcohol by vigorous shaking and thorough mixing. Separation of the organic phase was facilitated by centrifugation at 12,000xg for 10 minutes.

Complete spectra between $O.D_{450nm}$ and $O.D_{600nm}$ were obtained against a reagent blank. The MDA concentration of the sample can be calculated using an extinction coefficient of 1.56×10^5 m^{-1} cm^{-1} (Will, 1969). All results were expressed as nmol MDA per ml plasma.

As shown in Fig. 20 the curve was linear up to 20nmol/ml MDA when standard MDA concentration was plotted against absorbance at 532nm. However, the spike samples was not tested with the method.

2.3.2.3 CD ESTIMATION

CDs were assayed according to the method described in section 2.2.6.6.

2.3.2.4 FP ESTIMATION

FPs were estimated according to the method described in section 2.2.5.1.4.

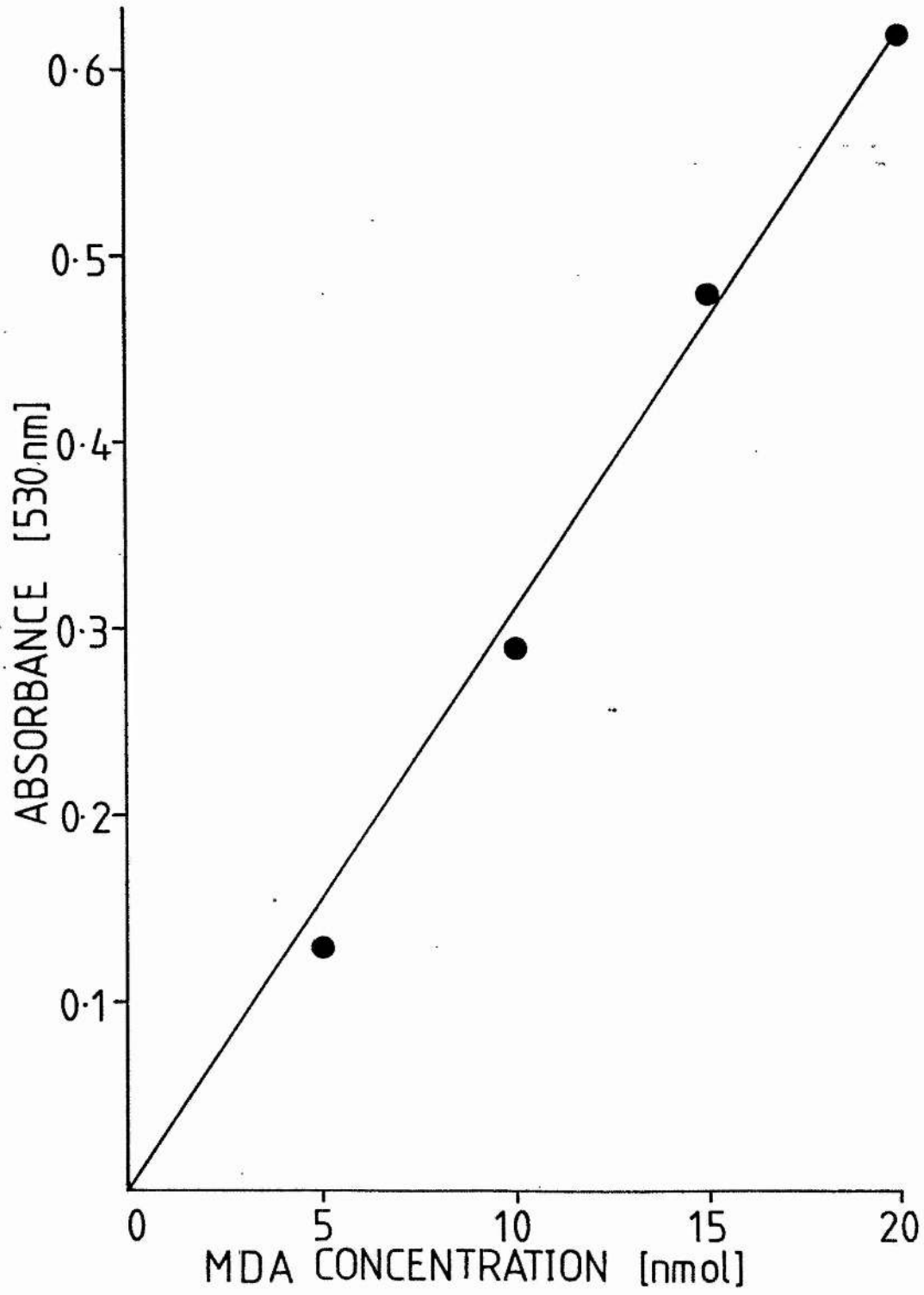
2.3.3 PLASMA STORAGE STUDIES

2.3.3.1 STORAGE PROCEDURE

Human plasma, stored at -20°C, (healthy+DMD) was obtained from previous studies. The samples to covered a range of storage periods 0.5 to 3 years. All plasma samples on thawing were clear and indistinguishable to the naked eye as to age. The samples used are listed in table (4) and (5) in previous section.

In addition, an experiment was carried out on sample from two healthy subjects to evaluate lipid peroxidation product levels during in 0.5 years of storage at -20°C.

Figure 20. Standard curve of malondialdehyde.



2.3.4 ANTIOXIDANTS IN HUMAN BLOOD PLASMA

2.3.4.1 BIOASSAY STUDIES - TOTAL ANTIOXIDANT ACTIVITY (AOA)

Total AOA was measured using an adaptation of the method of Stocks et al (1974).

2.3.4.1.1 ANIMAL SOURCE

Fresh ox-brain was obtained from St.Andrews slaughter house from freshly slaughtered animals and was transported to the laboratory packed in ice.

2.3.4.1.2 PREPARATION OF STANDARD HOMOGENATE

The ox brain was stripped of its meninges and all blood clots were washed off in ice-cold 0.15M sodium chloride. The tissue was then chopped into small pieces and homogenized for 2 min using a Potter Elvehjem type tissue grinder. in four times its weight of ice-cold PBS (40mM potassium dihydrogen phosphate/dipotassium hydrogen phosphate, pH 7.4 in 0.142M sodium chloride). The homogenate was centrifuged for 15min at 3500xg. The supernatant fluid was transferred to 20ml disposable containers. These were stored at -20^{o}C for up to 8 weeks.

2.3.4.1.3 AUTOXIDATION

A sample of the stock brain homogenate was thawed at room temperature and immediately diluted with three times its volume of PBS. 5.0ml portions of the dilute homogenate were transferred into a series of containers. Samples (50µl) of plasma or PBS (control) were added. Aliquots for zero-time MDA estimations

were removed immediately. The containers were then transferred to a 37°C water bath and incubation was continued for exactly 1hour.

2.3.4.1.4 MDA ESTIMATION

MDA concentration in the zero-time and one hour aliquots were measured by the thiobarbituric acid reaction (Sinnhuber and Yu, 1958; Stocks and Dormandy, 1971; Stocks et al.,1974). 2.0ml TCA (280g/l) was added to 4.0ml of homogenate and the precipitated protein removed by centrifugation. Then 4.0ml supernatant was heated with 1.0ml of 10g/l (w/v) thiobarbituric acid for 15min at 100°C. Absorbance was measured at 532nm. Results were calculated as nmol MDA per ml plasma.

2.3.4.1.5 CALCULATION OF ANTIOXIDANT ACTIVITY

The antioxidant activity of plasma was expressed in terms of percentage inhibition of spontaneous autoxidation as measured in the control homogenate. The calculation was based on the following equation:

$$AOA = 1 - \frac{\text{nmol of MDA/ml } (T_1) - \text{nmol of MDA/ml } (T_0)}{\text{nmol of MDA/ml } (C_1) - \text{nmol of MDA/ml } (C_0)} \times 100$$

C_0 = Control at 0 time

C_1 = Control at 1 hour

T_0 = Test at 0 time

T_1 = Test at 1 hour

2.3.4.2 VITAMIN E ESTIMATION

2.3.4.2.1 STANDARD ALPHA-TOCOPHEROL

A stock solution of 1mg/ml was dissolved in methanol and stored in dark bottles at -20°C. The working standard was prepared by diluting stock solution (1:50 v/v) in methanol (final concentration 20mg/l), stored in dark bottles at -20°C and never exposed to natural illumination. Working standard was prepared fresh every 2 weeks.

2.3.4.2.2 INTERNAL STANDARD (TOCOL)

Tocol was a gift of Roche Pharmaceuticals (Welwyn Garden City, Hertfordshire AL7 3AY, England) and was obtained as a stock solution in methanol (approximately 2mg/l). The solution was stored in a dark container at -20°C. The working internal standard was prepared by diluting stock internal standard (1:50 v/v) in methanol. The working internal standard was stored in dark bottles at -20°C and under these conditions, it was stable indefinitely. The calibration solution (1:10 v/v) was preparing by diluting the working internal standard (100:900 v/v) in methanol. The calibration solution was prepared fresh for each estimation.

2.3.4.2.3 CALIBRATION STANDARD

Equal volumes of alpha-tocopherol (20mg/l) and tocol (1:10 v/v) stored at -20°C in dark container. They were prepared fresh every couple of days.

2.3.4.2.4 EXTRACTION PROCEDURE

Duplicate 500µl aliquots of plasma were pipetted into pre-washed (methanol then with hexane) glass extraction tubes. To each tube 25µl working tocol standard (1:50 v/v), was added with a a micro syringe and mixed thoroughly with a vortex mixer. While the tube contents were mixed on a vortex mixer, 500µl ethanol were added slowly to deproteinize the sample. Then 2.0ml hexane (HPLC grade) were added to extract the sample followed by mixing with a vortex mixer for 1 minute continuously. The tube contents were centrifuged for 5 minutes at 12,000xg. After centrifugation, the supernatant was transferred to a conical 10x100mm glass tube and the aqueous layer re-extracted with 2.0ml hexane for 1 minute as before. The combined organic extracts were evaporated at 40°C under a stream of nitrogen on a Dri-Block. The residue was re-dissolved in 500ul methanol and centrifuged for 5 minutes at 12,000xg. Then the methanol extract was stored in a dark container at -20°C until analysis by HPLC.

2.3.4.2.5 HPLC CONDITIONS AND ANALYSIS

2.3.4.2.5.1 INSTRUMENTATION

HPLC MODEL

A GILSON liquid chromatograph equipped with a Model 702 Gradient Manager, Model 802 Monometric Module, Model 303 liquid delivery module, in conjunction with a Model HM/HPLC Holochrome U.V-VIS detector were used for this study.

CHROMATOGRAPHY COLUMN

Stainless steel column packed with reversed-phase ZORBAX ODS (C_{18}) (particle size 5-6um) 4.6mm i.d x 2.5cm supplied by DuPont instruments, Wellington, Delaware.

MOBILE PHASE, FLOW RATE AND PRESSURE

Elution was performed with methanol at a flow rate of 2ml per minute (pressure = 2000 PSI).

DETECTOR WAVELENGTH

The column effluent was monitored at 292nm at a sensitivity setting of 0.02.

GUARD COLUMN

A Du-Pont stainless steel column (4.6mm i.d x 5cm) packed with ODS was used.

RECORDER

Shimadzu Data Processor Chromatopac C-RIB was employed (Shimadzu Cooperation, Analytical Instruments Plants, Kyoto, Japan).

2.3.4.2.5.2 HPLC ANALYSIS

100ul of the sample was injected onto the top of column using a syringe loading sample injector (Model 7125; from Rheodyne Incorporated, Calif, USA). The absorbance at 292nm was measured at a chart speed of 0.5cm per minute. Peak areas were

automatically obtained by means of a Shimazu Data Processor Chromatopac. Instrument was calibrated with an equal volume of alpha-tocopherol (20mg/l) and tocol (1:10 v/v) prior to each run. The instrument is capable of printing chromatogram (waveform) and processed data (values in mg/litre alpha-tocopherol)) on the same recording chart.

2.3.4.3 DETERMINATION OF CAERULOPLASMIN AND TRANSFERRIN

Quatitative radial immunodiffusion and rocket (Laurell) immunoelectrophoresis were used to estimate caeruloplasmin and transferrin according to the method of Kemp (1983, personal communication).

2.3.4.3.1 BUFFER PREPARATION

Buffer for agar and electrophoresis was prepared as followed: 4.7g tris were dissolved in distilled water, 5.0ml 1M phosphoric acid added and the mixture made up to 1 litre with distilled water. Then, the pH was adjusted to 8.6.

2.3.4.3.2 AGAROSE PREPARATION

1.0g agarose was soaked in 50ml water and brought carefully to the boil. Shortly after boiling, when all the agar had dissolved, 0.05g sodium azide were added and the agar dispersed into 5ml aliquots, which were stored at $4^{o}C$ and remelted as required.

2.3.4.3.3 METHODS

2.3.4.3.3.1 RADIAL IMMUNODIFFUSION

5.0ml of agar were melted and 5.0ml phosphate buffer pH

8.6 (prewarmed to 50°C) were added. The mixture was mixed thoroughly and left at 50°C for 10 minutes. Then 1ml 10% human anti-serum (reconstituted anti-transferrin - was allowed to stand for 1 hour at room temperature before use) was added and poured immediately after thoroughly mixing (as quickly as possible but avoiding air bubbles) onto a plastic immunodiffusion plate. The plate was left for 30 minutes to allow the agar to solidify. Holes (or wells) were punched in the solidified agar with a hole puncher (medium size) about 2mm in diameter and approximately 2cm apart. The wells were filled with 10µl plasma samples (undiluted samples for caeruloplasmin; 1:2 samples for transferrin). Different dilutions of a standard plasma solution was used to plot a standard curve (dilutions: 1:1, 1:2, 1:3, and 1:4). The remaining wells were used for the samples being analyzed. Each well was filled with samples (undiluted samples for caeruloplasmin analysis but 1:2 dilution for transferrin analysis); the diluent was phosphate buffer pH 8.6. Then the plates were left in a moisture chamber at room temperature. After, 48 hours the diameter of circular immunoprecipitates in the gel layer were measured using MacSweeney ruler against a dark background. Standard curve were obtained by plotting the squares of the standard control diameters (mm) against antigen concentration. The diameters of the immunoprecipitin rings of the test samples were then measured. The corresponding protein concentrations were read from the straight line standard curve. Multiplied by the dilution factors, these give the concentration of each protein in the undiluted specimen.

2.3.4.3.3.2 ROCKET (LAURELL) IMMUNOELECTROPHORESIS

5.0ml of agar were melted and 5.0ml of phosphate buffer pH 8.6 (prewarmed to 50°C) was added. The mixture was mixed thoroughly and left at 50°C for 10 minutes. Then a quatity for antiserum (3% for anti-transferrin and 5% for anti-caeruloplasmin) was added. The mixture was mixed thoroughly and poured immediately onto scrupulously clean glass plates (10x10cm) resting on an absolutely level surface. After 15 minutes, the solution solidified to a transparent gel of uniform thickness (2.3-2.4mm). After cooling wells were punched about 2.0mm diameter. The distance between adjacent wells (sites) was 1.0cm and they were 1.5cm from the edge. A well puncher was used to ensure uniformity of size and shape. The samples (10µl) were introduced into the wells. Four different dilutions of normal control standard plasma were also included as a requirement for the plotting of a standard curve (dilutions 1:1, 1:2, 1:3, and 1:4). The slides were then placed immediately in the electrophoresis tanks (Shandon). The wells were located on the cathode side and the conditions for electrophoresis were as follows:

BUFFER : Phosphate buffer pH 8.6

Contact between buffer and gel plate: filter paper strips moisturised with buffer solution.

VOLTAGE : 150volts between the ends of the plate.

DURATION : electrophoresis was carried out for at least 3 hours.

The formation of immunoprecipitin peaks during electrophoresis can readily be observed against a dark background. The process of electrophoresis was complete when the tips of all the peaks ("rocket") were closed. The precipitin peaks were measured (i.e length of peak from point of application to tip) immediately after completion of the electrophoresis. A plate (10x10cm) can carry up to 7 antigen wells. At least three of these wells were filled with normal control standard solution. When the length of the standard peaks were plotted against the concentration of the standard solutions, the resulting curve could be used to determine the protein content after multiplication by corresponding dilution factor.

2.4 STATISTICAL ANALYSIS

Where possible all measurements in this study were made in triplicate. From these triplicate the mean was derived. Each mean value plotted on a graph or histogram is given with its range (that is with the lowest and highest value).

To test the difference between the means of two small samples, the population variances were assumed to be equal and student's t-statistic was calculated as follow:

$$t = \frac{\bar{x}_1 - \bar{x}_2}{s\sqrt{(1/n_1 + 1/n_2)}}$$

where $\bar{x}$ is a sample mean, n is the number of samples and s is the standard deviation.

The standard deviation was derived as follows:-

$$s^2 = \frac{1}{n_1 + n_2 - 2}\left(\sum x_1^2 - \frac{(\sum x_1)^2}{n_1} + \sum x_2^2 - \frac{(\sum x_2)^2}{n_2}\right)$$

In practice a computer program (G. Kemp, personal communication) was used to calculate t and the significance of the difference between the two samples deduced from standard statistical tables for students t-test.

To test the correlation between two set of measures, a scatter diagram showing the association was plotted and the

following relationship was evaluated using a computer program (G. Kemp, personal communication).

$$r = \frac{\sum (x_i - \bar{x})\ (y_i - \bar{y})}{\sqrt{\sum (x_i - \bar{x})^2\ \ (y_i - \bar{y})^2}}$$

The statistic r was compared with values given in standard tables to obtain a linear relationship (positive or negative) between two set of measures being significantly different (Parker, 1973).

3 RESULTS

3.1 LIPID PEROXIDATION IN CSFs

Preliminary studies to determine the formation of MDA in CSFs in culture were done in two sets of experiments. In the first set, to determine endogenous levels of lipid peroxidation, CSFs were incubated in a medium without added PUFA (arachidonic acid, 20:4). And in the second set, CSFs were incubated in a medium with 120μM 20:4. The 20:4 concentration (120μM) was chosen for these studies because in a previous study it was demonstrated that CSFs have a capacity to produce MDA when incubated with this concentration of exogenous PUFA (Gavino et al.,1981). In addition, Asakawa and Matsushita (1980) found that 20:4 is very easily oxidized and yields a very high colour intensity of MDA as measured by TBA test.

Each data set is derived from three different lines of CSFs. The control line was HAM 2 and the DMD lines, 1 and 2 in following section, were HAM 1 and HAM 5 (see section 2.2.2.2 for details). All cells were grown in T_{25} ($25cm^2$) flasks.

Each experiment involved the use of ten T25 flasks of CSFs for each line. For MDA measurement, each line was treated in two ways. One batch of flasks were not washed with phosphate buffer saline (PBS)(called unwashed cells), whilst a second batch of CSFs were washed twice with PBS (called washed cells), before reaction with TBA.

MDA production was measured by the method reported by Gavino et al (1981) as described under "Material and Methods". It was observed

that MDA production in the normal CSFs was usually strikingly less than that in either of the DMD lines whatever the incubating conditions or times. Results of these are summarised in Table 6 and 7.

3.1.1 LIPID PEROXIDATION PRODUCTS IN WHOLE CONFLUENT CSFs

3.1.1.1 UNSUPPLEMENTED EXPERIMENTAL MEDIUM

3.1.1.1.1 UNWASHED UNSUPPLEMENTED CSFs

Production of TBA-reactive material is shown in Table 6 and Fig. 21. The results show similar increased MDA production in DMD CSFs compared to normal control CSFs at all times of sampling. The absolute amount of MDA measured in the control line at all time intervals was less than that in either of the DMD lines. However, in terms of percentage increase between 24 h (the earliest time interval used at which TBA-reactive material was detectable) and 96 h, the control line showed the greatest change (217%) whereas the DMD lines showed more modest increases (74% and 98%).

Table 6. Malonyldialdehyde concentration (nmol/flask) in washed and unwashed cells from normal (HAM2) line and DMD lines (HAM1 and HAM5) after varying time of incubation in medium without PUFA.

Cell Lines \ Data		TIME (hours) 0	24	48	72	96
		X±S.E	X ±S.E	X ±S.E	X ±S.E	X ±S.E
Normal (HAM2)	Unwashed	0±0	0.12±0.01	0.24±0.10	0.34±0.14	0.38±0.13
	Washed	0±0	0.28±0.11	0.46±0.13	0.59±0.23	0.62±0.16
DMD (HAM1)	Unwashed	0±0	1.00±0.30	1.89±0.20	1.90±1.30	1.98±1.34
	Washed	0±0	0.48±0.20	1.25±1.08	3.25±0.98	4.25±2.90
DMD (HAM5)	Unwashed	0±0	1.14±0.25	1.58±1.01	1.83±0.83	1.98±0.76
	Washed	0±0	0.51±0.10	2.38±1.00	3.50±2.14	3.98±1.41

Table 7. Malonyldialdehyde concentration (nmol/flask) in washed ans unwashed polyunsaturated acid incubated cells from normal line (HAM2) and DMD lines (HAM1 and HAM5) at varying time intervals.

Cell Lines / Data		TIME (hours) 0	24	48	72	96
		X±S.E	X ±S.E	X ±S.E	X ±S.E	X ±S.E
Normal (HAM2)	Unwashed	0±0	2.36±0.54	2.40±0.54	2.48±0.51	2.50±0.41
	Washed	0±0	1.99±0.80	2.39±0.28	3.28±0.66	4.88±2.60
DMD (HAM1)	Unwashed	0±0	0.61±0.14	1.72±0.84	3.44±0.65	8.48±1.43
	Washed	1.96±1.47	3.26±1.17	5.76±2.50	9.28±3.98	13.81±5.42
DMD (HAM5)	Unwashed	0±0	1.56±0.18	2.64±0.18	3.48±0.25	3.84±1.23
	Washed	1.68±1.44	2.81±0.18	5.64±0.14	11.8 ±2.80	16.5 ±3.28

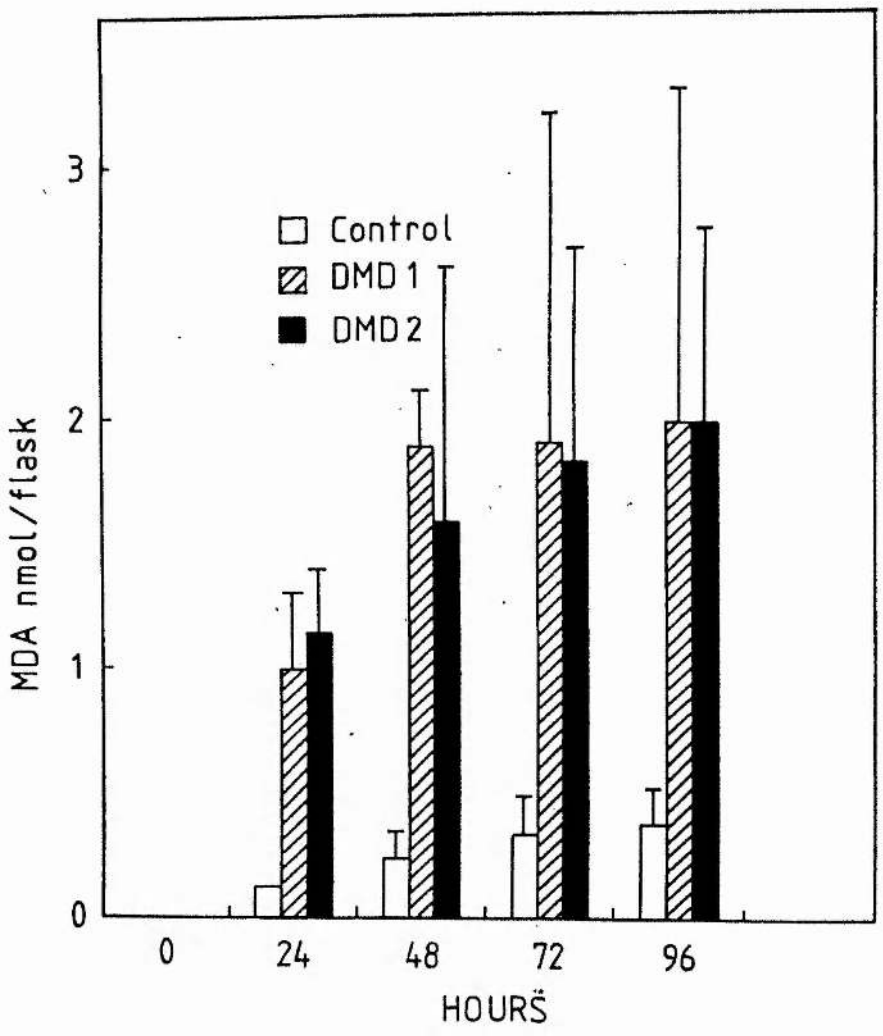


Figure 21. TBA-reactive material in human skin fibroblasts from Duchenne patients and a normal control (unwashed cells, no PUFA).

3.1.1.1.2 WASHED UNSUPPLEMENTED CSFs

TBA-reactive materials were estimated in whole cultures in exactly the same way and during simultaneous incubations as for 3.1.1.1 but with the confluent monolayer being washed with PBS immediately prior to TCA addition and colour development. Table 6 and Fig. 22 show several important effects of this washing procedure:-

(i) The absolute amounts of MDA generated in the DMD cultures is much greater (approx. twofold) by the end of the incubation period than in unwashed cells whereas that in the control cultures is not so markedly increased.

(ii) As for unwashed cells, and particularly by 72 and 96 h, the concentration of MDA is consistently higher in DMD lines than that in control.

(iii) The percentage increase in MDA production at 96h compare to 24h is much greater in washed rather than unwashed DMD cells (DMD 1=785%, DMD 2=680%) whereas there is less increase in control washed cells (121%) compared with unwashed.

Although there are differences in MDA production between the two DMD lines in unwashed but more particularly in washed cells nevertheless these are not nearly as great as those between the control line and the DMD cells.

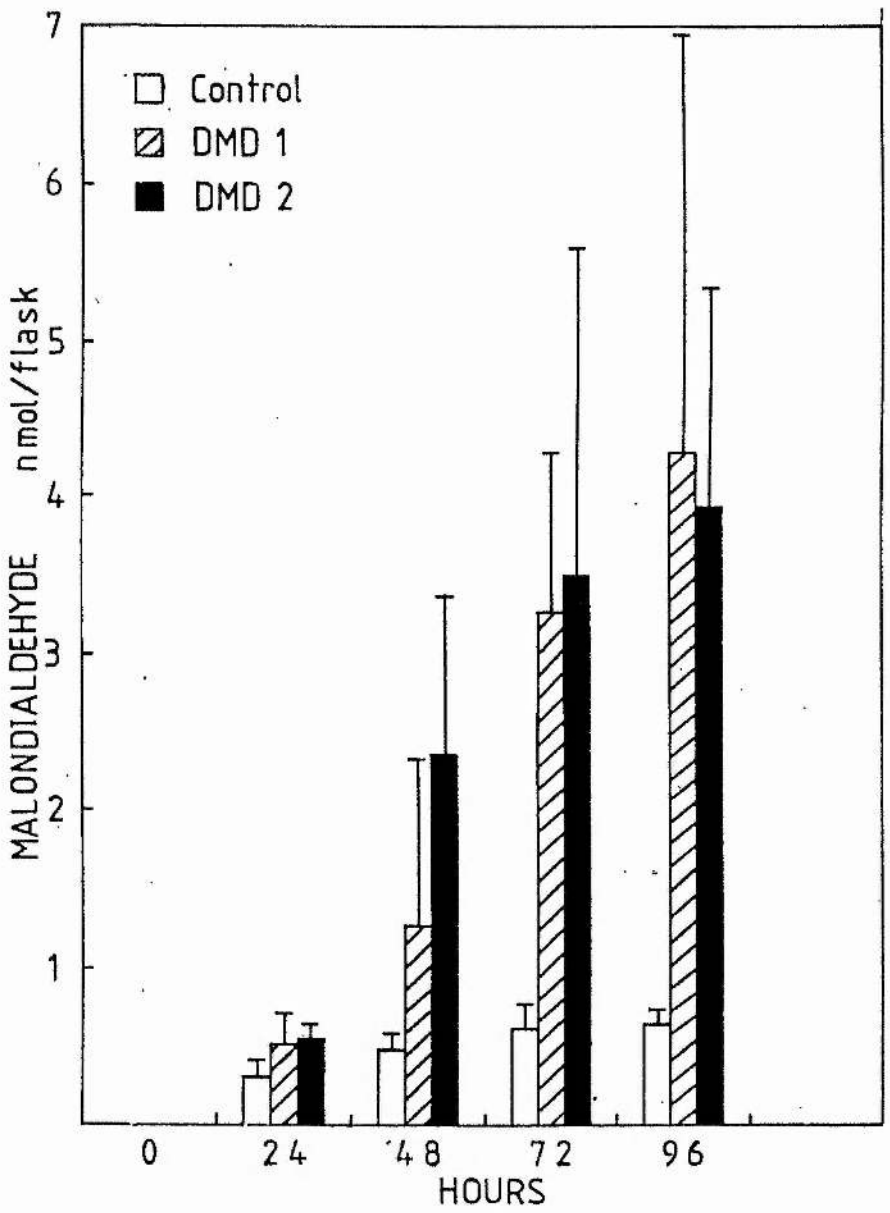


Figure 22. TBA-reactive material in human skin fibroblasts from Duchenne patients and a normal control (washed cells, no PUFA).

3.1.1.2 LIPID PEROXIDATION IN CELLS GROWN IN MEDIUM SUPPLEMENTED WITH 120 μM ARACHIDONIC ACID (20:4)

Since another group (Gavino et al.,1981) has reported the stimulatory effect of PUFA on MDA production in confluent human CSFs from normal subjects, using 120μM in culture medium, it was decided to use the same concentration with our cells to see whether addition of such a stressor might accentuate even further the differences between DMD and normal lines. This PUFA is easily peroxidised and will initiate a chain reaction of further peroxidation if unchecked by protection mechanisms. Incubation conditions and assay of TBA-reactive materials were otherwise exactly as in 3.1.1.1

3.1.1.2.1 UNWASHED CELLS + 20:4

Contrary to results with unsupplemented media little difference was observed between MDA production in unwashed, normal and DMD lines, except after 96 h when one of the DMD lines particularly showed a striking increase (Fig. 23 and Table 7) amounting to more than a doubling in MDA between 72 and 96 h when compared with the levels measured in all three lines at 72 h. Although the difference between normals and DMD is not so pronounced in unwashed, 20:4 supplemented cells, the absolute levels of MDA are much higher than unwashed cells without added 20:4, (maximum 2nmol/flask).

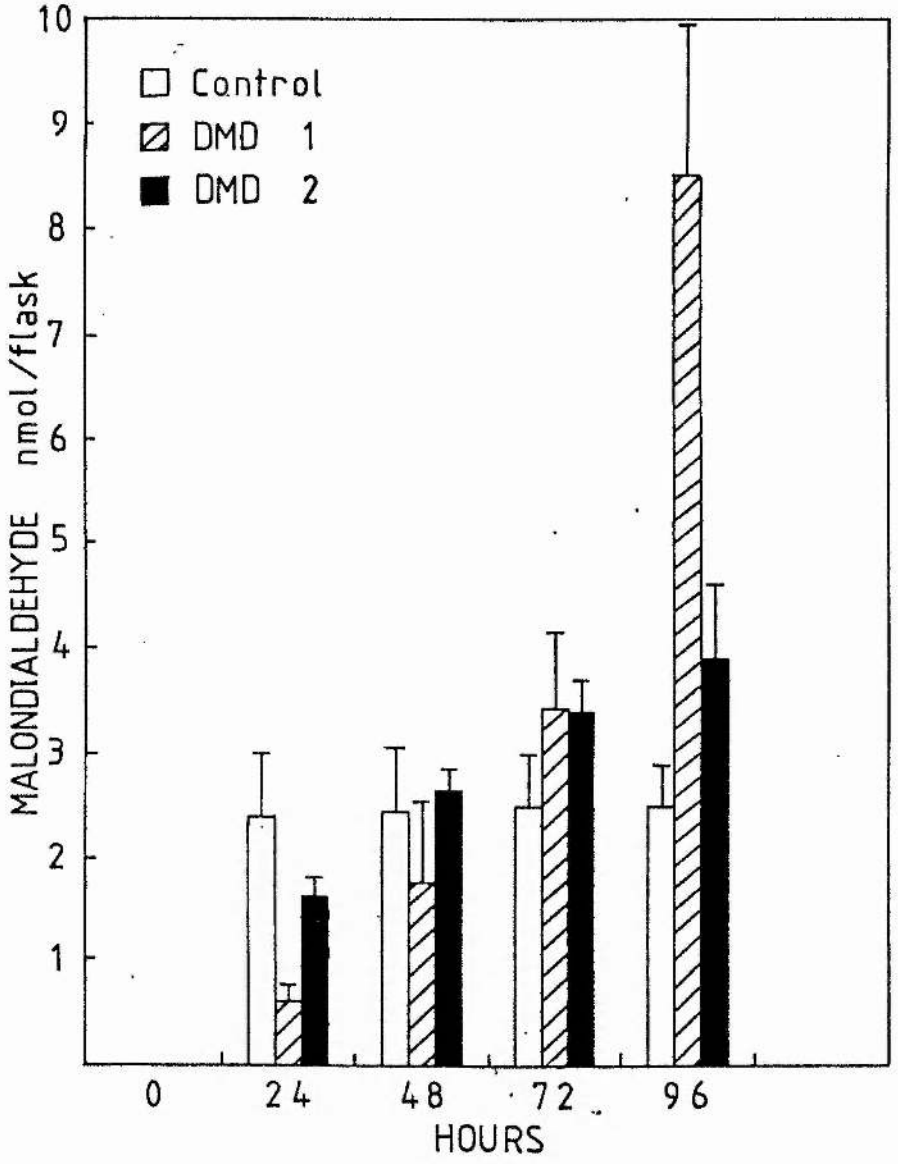


Figure 23. Effect of PUFA (20:4 ; 120µM) on TBA-reactive material (as MDA) of human skin fibroblasts (unwashed cells).

3.1.1.2.2 WASHED CELLS + 20:4

Table 7 and Fig. 24 show the results obtained. Interestingly in this experiment, MDA was measureable at zero time of incubation (estimated just after adding fresh supplemented culture medium to confluent cells) in both DMD 1 and 2 (1.96 ± 1.47 and 1.68 ± 1.44 nmol/flask respectively) whereas at the same time under these assay conditions MDA was undetectable in the control line. Fig. 24 shows clearly that MDA production increases markedly with time in the DMD lines (MDA at 96 h is approx. 8-fold that at zero time) whereas the increase for control cells over the 24-96 h time period is much more modest (2.5-fold).

3.1.1.3 EFFECTS OF WASHING AND 20:4 SUPPLEMENTATION ON LIPID PEROXIDATION IN CSFs

Comparing the data obtained from parallel experiments with the one control and two DMD cell lines it is possible to draw the following general conclusions (albeit based on a small number of lines) (see Figs. 21, 22, 23, 24).

(i) washing either supplemented or unsupplemented cultures with PBS (removal of an extracellular antioxidant ?) immediately prior to MDA estimation has a pronounced effect, particularly after 24 h incubation, on the final concentration measured. In general, for a given medium washing as much as doubles the measureable TBA-reactive material (compare Figs. 21 and 22; 23 and 24)

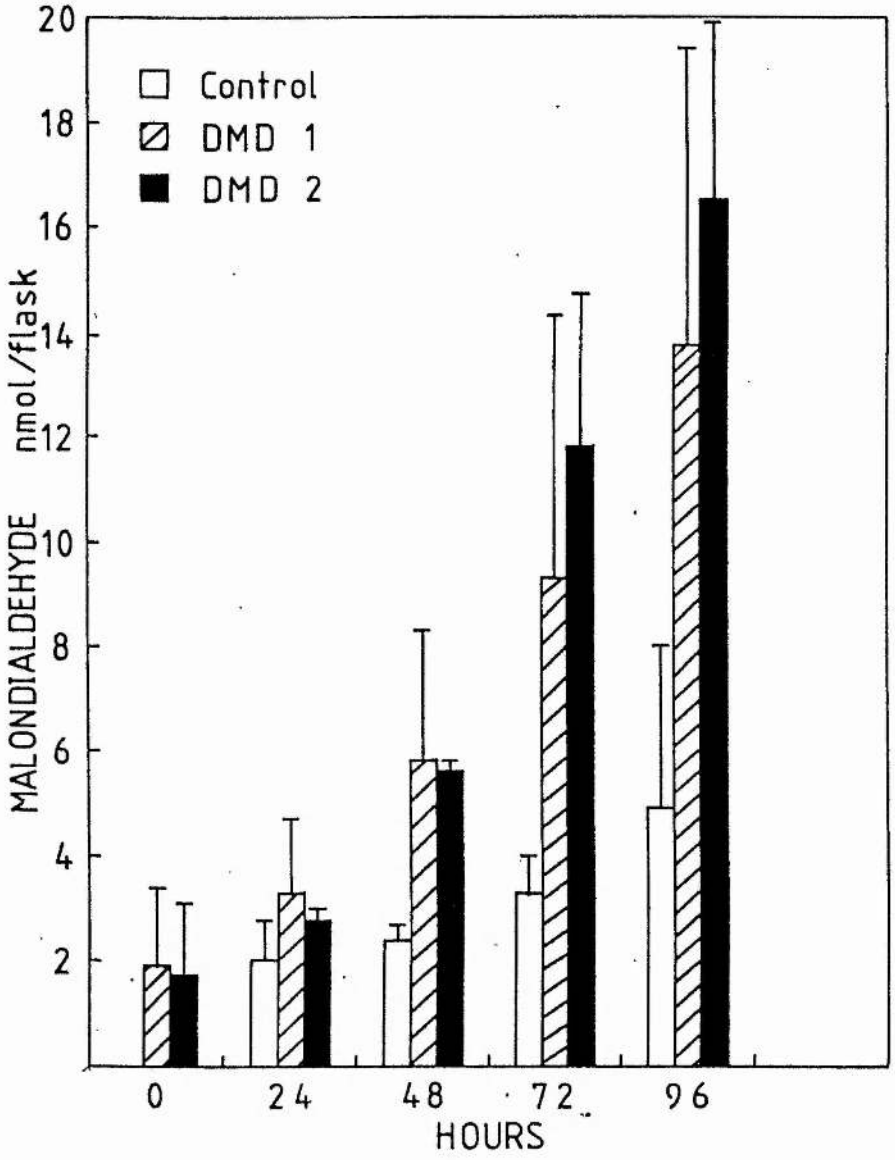


Figure 24. Effect of PUFA (20:4 ; 120µM) on TBA-reactive material (as MDA) of human skin fibroblasts (washed cells).

(ii) the presence of 20:4 undoubtedly increases the total measureable MDA, again particularly at the later incubation times and this effect is much more evident for washed cells (compare Figs. 21 and 23; 22 and 24)

Possible reasons for these effects are dealt with in the discussion.

3.1.1.4 COMPARISON OF NORMAL AND DMD LINES

Although the data is limited to one control and two DMD lines, there is a striking trend, under all sets of experimental conditions employed in these studies, for the two DMD lines to yield similar increased levels of TBA-reactive material when compared with the control line.

3.1.1.5 LIPID PEROXIDATION IN CONFLUENT CELLS GROWN WITH ADDED t-BUTYL HYDROPEROXIDE (TBH)

Lipid peroxidation has many deleterious effects on membrane structure and function (Vladimirov et al.,1980). Peroxidizing lipids generate many potentially cytotoxic products (Logani and Davies, 1980); stoichiometrically the major products are lipid hydroperoxides. Lipid hydroperoxides are highly toxic in vivo (Horgan et al.,1957) and in vitro are capable of inactivating enzymes (Chio and Tappel, 1969; Neukom, 1980), covalently altering biomolecules (Lewis and Wills, 1962) and initiating free-radical mediated destruction of proteins (Logani and Davies, 1980) and unsaturated lipids (Shimasaki and Privett, 1975). Based on these observations and the the work of Gavino et al (1981) we also made an attempt to measure MDA production in the same cell lines exposed to TBH (300µM). Externally added TBH may qualify as a model for endogenous lipid hydroperoxides (Sies and Summer, 1975). Unfortunately, after about 12 h of incubation with this final concentration (300µM) of hydroperoxide most of the CSFs (especially DMD lines) started to detach from the culture flask. As a result, the MDA concentrations measured were effectively meaningless as the cells were clearly being killed or severely injured by this concentration of TBH. The results have therefore not been included in this report. The clear conclusion from these observations is that this final concentration (300µM) of TBH is not suitable for oxidatively challenging human CSFs since it is obviously toxic to the cells. For this reason, another experiment was conducted, to determine the effect of different final concentrations

of TBH on cell growth and viability over the chosen period of incubation. The range of final TBH concentrations used were: 0, 25, 50, 75, 100, 150, 200, 250 and 300μM. The cells in each flask were examined by light microscopy immediately after adding TBH to the flask and continued for 96 h of incubation. It was found that the maximum concentration of TBH which allowed normal cell growth was 50μM. At this final concentration, the cells remain stuck down to the bottom of the culture flask and divide normally even up to 96 h of incubation. But final concentrations above 50uM seem unsuitable for studies with human CSFs since it was observed that the cells started to detach as early as 6 h after addition of concentrations of TBH more than 50μM. Unfortunately, contamination problems prevented further CSF experiments at this stage and for the remainder of the project but it would be of great interest to test the effect of non-toxic (50μM ?) concentrations of TBH on normal and DMD lines.

3.1.2 LIPID PEROXIDATION BY WASHED PARTICULATE FRACTIONS FROM CSFs

In this study, the washed particulate fraction from CSFs was used to investigate whether the observed increased lipid peroxidation in DMD cells is solely a membrane-dependent process or interrelated with other cell components. Two lipid peroxidation products, namely MDA and FP, after induction of lipid peroxidation via NADPH oxidase by Fe^{3+}/ADP/NADPH, were studied. Fig. 25 and Table 8 summarises MDA production when the reaction was stopped at various times of incubation. Clearly MDA concentration increases significantly with time of incubation only in the case of NADPH stimulated cells. Two important points emerge from this data:

(i) the endogenous levels of MDA at zero time are very similar in control and DMD fractions;

(ii) thereafter there is no appreciable differences between control and DMD, with or without NADPH added.

Table 8. Effect of NADPH on lipid peroxidation in particulatefraction from cultures skin fibroblasts from patients with DMD (n = 1) and normal control subject (n = 1).

Incubation time (minutes)	MALONDIALDEHYDE nmol mg^{-1} Protein				FLUORESCENT PIGMENT Relative fluorescence units mg^{-1} Protein			
	ENZYMIC SYSTEM				ENZYMIC SYSTEM			
	-NADPH		+NADPH		-NADPH		+NADPH	
	NORMAL	DMD	NORMAL	DMD	NORMAL	DMD	NORMAL	DMD
0	1.9±0.2	2.1±0.3	1.9±0.1	2.1±0.3	26.1±1.6	48.0±3.2	26.1±1.6	48.0±3.2
60	1.9±0.2	2.3±0.2	4.4±0.3	4.6±0.8	38.0±3.2	98.0±4.7	140.0±6.3	150.0±12.6
120	2.0±0.4	2.3±0.2	4.6±0.3	4.6±0.4	86.0±3.2	101.0±4.7	146.0±7.9	233.0±9.5
180	2.2±0.2	2.7±0.3	4.6±0.4	5.3±0.7	89.0±4.7	122.0±4.7	164.0±6.3	249.0±4.8

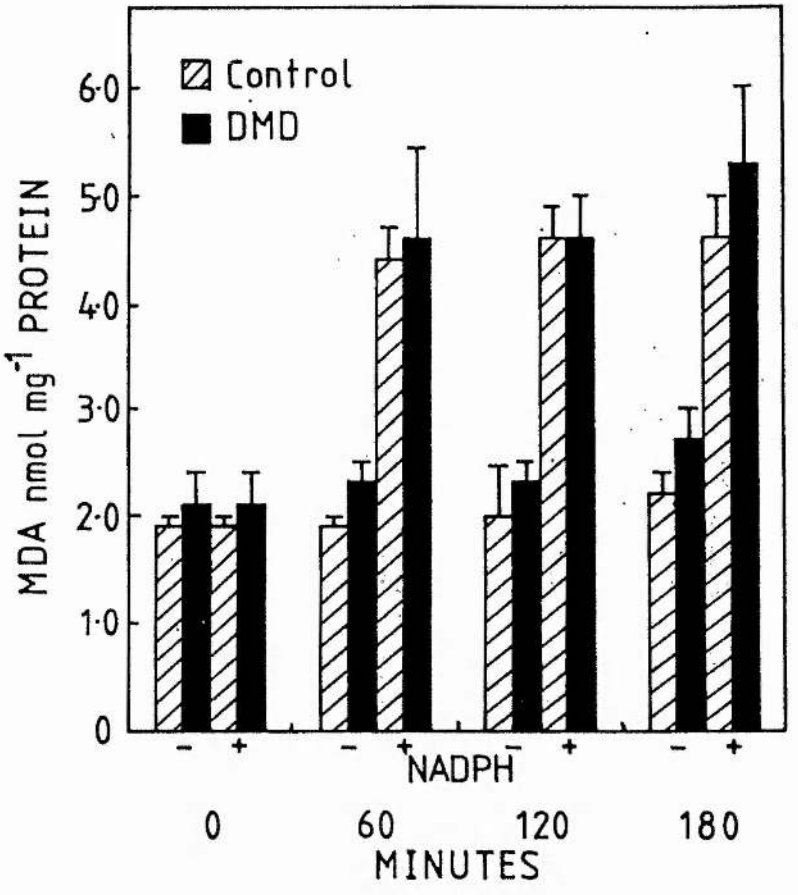


Figure 25. Enzymic lipid peroxidation at various times of incubation in normal control and DMD human skin fibroblast particulate fraction measured by TBA-reactive material (nmol MDA/mg protein).

The zero time FP levels are lower in control fraction than in DMD (Fig 26) and with increasing incubation time there is a significant increase in NADPH-stimulated DMD fractions compared to controls although, when calculated on the basis of percentage of zero time values, there is no significant difference. As expected there is a marked increase in FP with time in the incubations containing NADPH, particularly in the first 60 minutes.

Clearly from these observations, there is no significant difference in enzyme-induced lipid peroxidation, as measured by MDA and FP, between DMD and normal control CSF particulate fractions. These results prompted us to study the possibility that cytoplasmic components might also be involved in propagation or potentiation of lipid peroxidation. Whole homogenates of CSFs from DMD and normal control lines were used for this purpose and these results are presented in the next section.

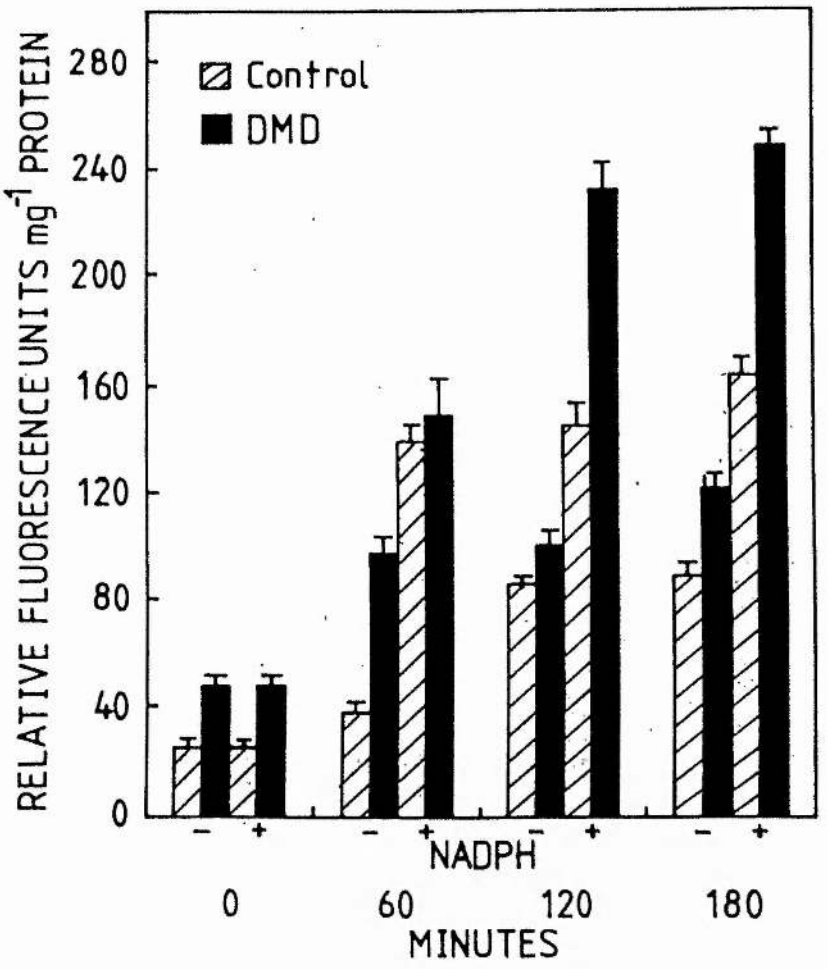


Figure 26. Enzymic lipid peroxidation at various times of incubation in normal control and Duchenne human skin fibroblast particulate fraction measured by fluorescent pigment concentration (relative fluorescence units/mg protein).

3.1.3 LIPID PEROXIDATION IN UNFRACTIONATED HOMOGENATES OF CSFs

Homogenates of CSFs from normal (n=6) and DMD (n=2) (Table 3) lines formed lipid peroxides as measured by U.V absorbance (conjugated diene, CD), TBA test (MDA), and FP, when incubated at 37°C in both the enzymic system (Fe^{3+}/ADP/NADPH) and the non-enzymic system (Fe^{3+}/ascorbate) at pH 7.0.

As shown in Table 9 and Figs. 27-28 the concentration of CD at zero time is higher in DMD CSFs homogenates in both systems, compared to normal controls. Figs. 27-28 show that the CD value increased more rapidly in the non-enzymic than enzymic system. However, both systems consistently show a tendency for the value to reach a peak at 120 minutes of incubation which is essentially unchanged by further incubation. Enzyme-induced CD production expressed as a percentage of zero time value was almost identical in DMD compared with control homogenates, although the DMD preparations exhibited a consistently higher value. An interesting effect is seen with the non-enzymic system where, in the control (i.e. without ascorbate) incubations there is a striking increase in CD only in the DMD homogenates. When ascorbate is present, the difference between normal and DMD homogenates is only apparent at 0 and 60 minutes and vanishes after 120 and 180 minutes. By 180 minutes the CD levels are more or less identical in both ascorbate-stimulated and unstimulated incubations, which is surprising.

Table 9. Lipid peroxidation products in whole fibroblast homogenates from patients with DMD (n = 2) and normal control subjects (n = 6) induced by Fe^{3+}/ADP/NADPH (enzymic) and Fe^{3+}/ascorbate (non-enzymic).

Incubation time (minutes)	CONJUGATED DIENE A_{235} mg^{-1} Protein				MALONDIALDEHYDE nmol mg Protein				FLUORESCENT PIGMENTS Relative units mg^{-} protein			
	ENZYMIC SYSTEM				ENZYMIC SYSTEM				ENZYMIC SYSTEM			
	-NADPH		+NADPH		-NADPH		+NADPH		-NADPH		+NADPH	
	NORMAL	DMD	NORMAL	DMD	NORMAL	DMD	NORMAL	DMD	NORMAL	DMD	NORMAL	DMD
0	2.60±1.04	2.72±0.96	2.60±1.04	2.72±0.96	3.64±1.21	4.00±1.13	3.64±1.21	4.00±1.13	44.20±3.35	49.50±7.78	44.20±3.35	49.50±7.78
60	2.64±0.64	2.82±0.84	2.76±1.12	2.96±0.68	3.90±1.08	4.60±1.13	5.80±1.15	6.50±1.56	44.00±2.65	52.00±2.83	44.20±3.03	67.00±7.07
120	2.68±0.68	2.86±0.82	2.92±1.14	3.12±0.41	5.08±0.88	5.70±0.85	11.40±1.13	11.40±1.13	44.40±2.07	53.00±4.24	51.00±5.79	80.00±11.31
180	2.68±0.72	2.92±0.81	3.00±1.94	3.12±0.84	5.68±0.86	6.80±0.57	12.42±1.69	12.40±0.85	46.40±3.78	54.00±2.83	57.60±7.70	86.00±12.02
Incubation time (minutes)	NON-ENZYMIC SYSTEM				NON-ENZYMIC SYSTEM				NON-ENZYMIC SYSTEM			
	-ASCORBATE		+ASCORBATE		-ASCROBATE		+ASCORBATE		-ASCORBATE		+ASCORBATE	
	NORMAL	DMD	NORMAL	DMD	NORMAL	DMD	NORMAL	DMD	NORMAL	DMD	NORMAL	DMD
0	2.44±1.16	2.55±0.84	2.44±1.16	2.55±1.00	3.80±1.36	4.40±0.28	3.80±1.36	4.40±0.28	41.40±4.22	49.50±9.19	41.40±4.22	49.50±9.19
60	2.46±0.84	2.82±0.64	2.56±1.24	2.86±0.68	3.96±1.23	4.40±0.28	5.60±0.93	5.50±0.14	42.20±4.09	50.50±9.19	43.60±4.39	54.50±9.19
120	2.47±0.42	2.84±0.72	2.74±0.86	2.88±0.66	4.70±1.07	5.00±0.28	10.64±1.47	10.80±0.57	42.80±3.89	51.00±8.49	49.80±5.31	61.00±7.07
180	2.46±0.51	2.88±0.55	2.84±0.94	2.99±1.12	5.10±1.04	5.10±0.42	11.00±1.38	11.20±0.28	44.20±4.21	53.00±7.07	55.40±7.40	71.50±6.36

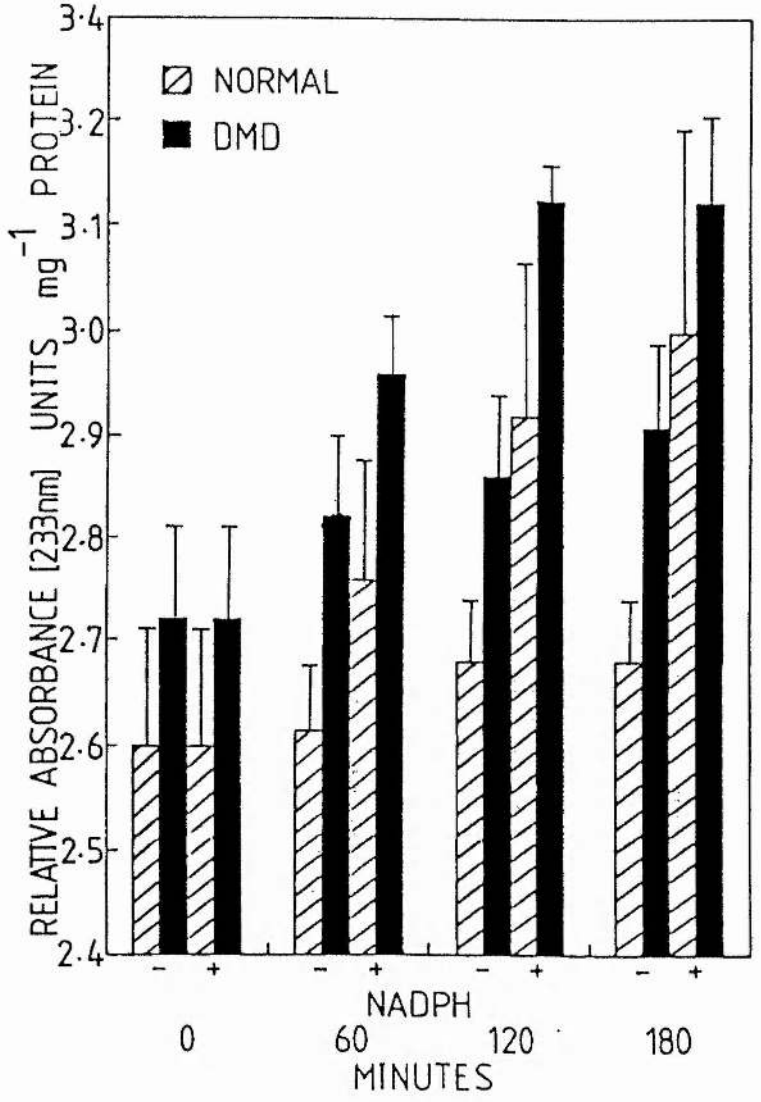


Figure 27. Conjugated diene produced by normal control (n=6) and DMD (n=2) whole fibroblast cell homogenates, expressed as relative absorbance (233nm) units/mg homogenate protein. (+) Fe^{3+}/ ADP/NADPH (0.12mM, 2mM and 4mM respectively) (-) Fe^{3+}/ADP only.

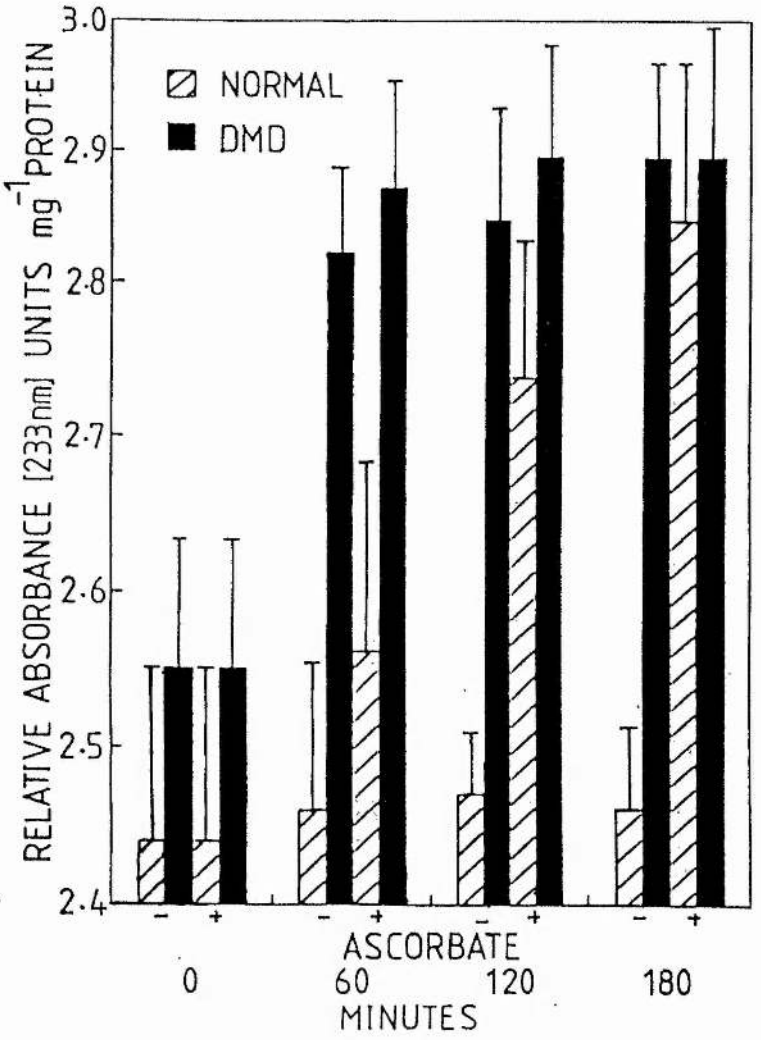


Figure 28. Conjugated diene produced by normal control (n=6) and DMD (n=2) whole fibroblast cell homogenates, expressed as relative absorbance (233nm) units/mg homogenate protein. (+) Fe^{3+}/ ascorbate (12µM and 0.66mM), (-) 12µM Fe^{3+}only.

Figs. 29 and 30 show the results for TBA-reactive materials in the same incubations. The basal level of MDA analyzed at experimental time zero was very similar in both homogenates in both systems. In the enzymic system, as expected, the MDA yield was higher in the NADPH-treated incubations but there was no difference between DMD and normal in either the presence or absence of NADPH. The significant rise in MDA occurred between 60 and 120 minutes for NADPH-treated preparations, whereas the bulk of the increase in CD for both non-enzymic and enzymic systems occurred both between 0-60 and 60-120 minutes.

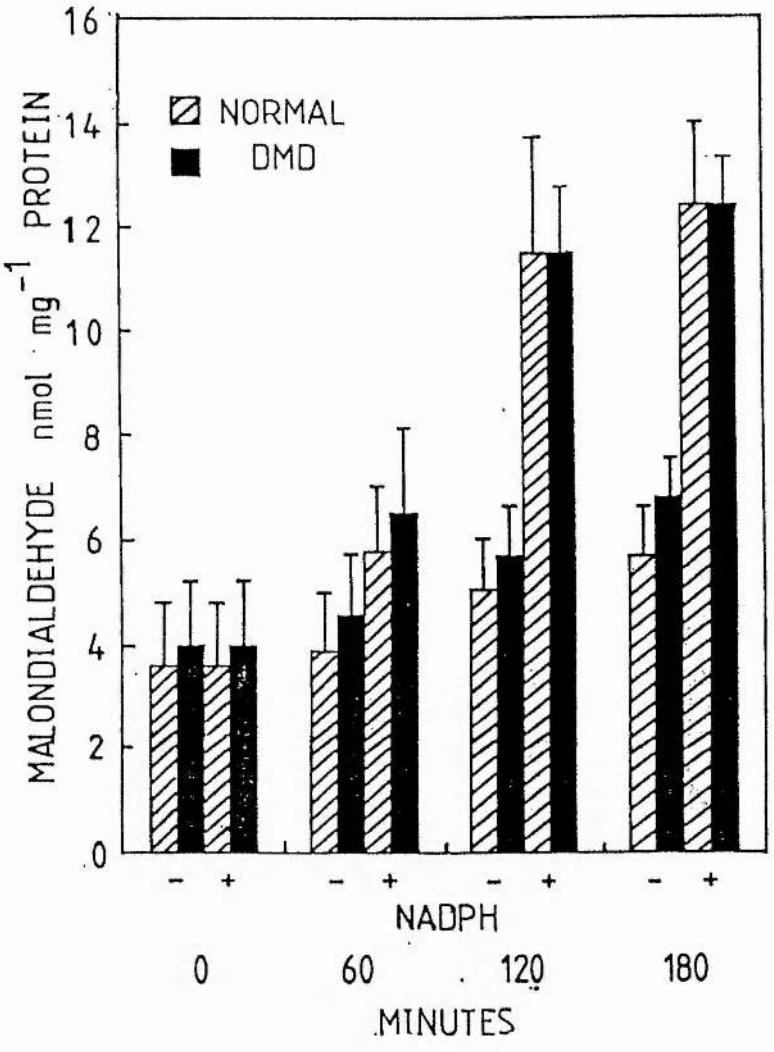


Figure 29. TBA-reactive material produced by normal control (n=6) and DMD (n=2) whole fibroblast cell homogenates, expressed as nmol MDA/mg homogenate protein. (+) Fe^{3+}/ADP/NADPH (0.12mM, 2mM and 4mM respectively), (-) Fe^{3+}/ADP only.

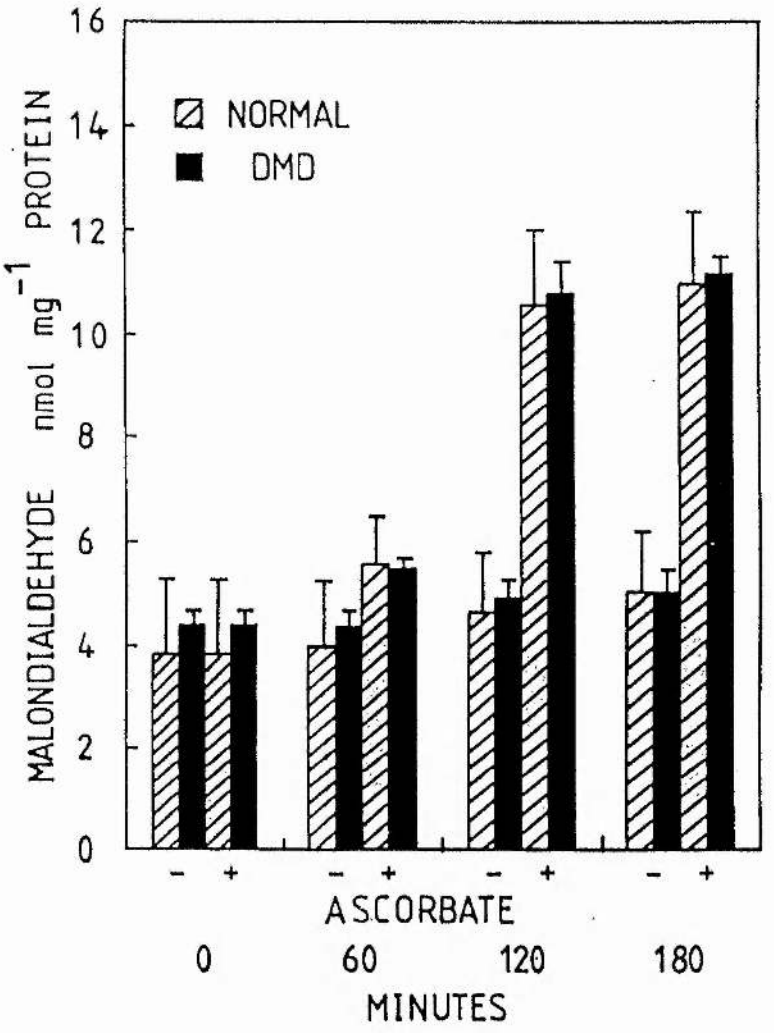


Figure 30. TBA-reactive material produced by normal control (n=6) and DMD (n=2) whole fibroblast cell homogenates, expressed as nmol MDA/mg homogenate protein. (+) Fe^{3+}/ascorbate (12μM and 0.66mM respectively), (-) 12μM Fe^{3+} only.

Lipid-soluble FP showed relatively small increases over the time period of incubation in both the enzymic and non-enzymic systems although the DMD homogenates again showed consistently higher concentrations irrespective of time or system (Figs. 31 and 32). The significance of the difference between DMD and normal is questionable however, with the exception of NADPH induced incubations where the effect is clear (Fig. 31).

To summarise, a significant difference between DMD and normal homogenates was seen for:-

(i) absolute concentrations of CD and FP under all conditions

(ii) CD in control (-ascorbate) non-enzymic incubations.

(iii) FP in NADPH-induced (enzymic) incubations.

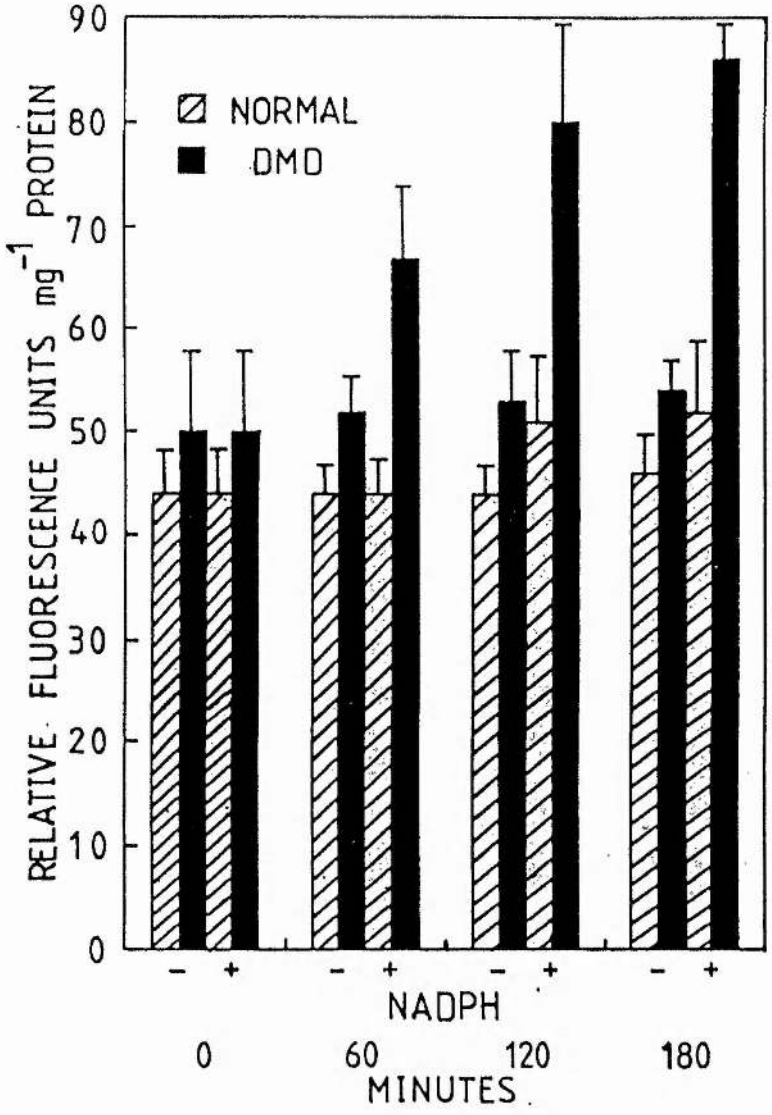


Figure 31. Fluorescent pigments produced by normal control (n=6) and DMD (n=2) whole skin fibroblast cell homogenates, expressed as relative fluorescence units/mg homogenate protein. (+) Fe^{3+}/ADP/NADPH (0.12mM, 2mM and 4mM), (-) Fe^{3+}/ADP only.

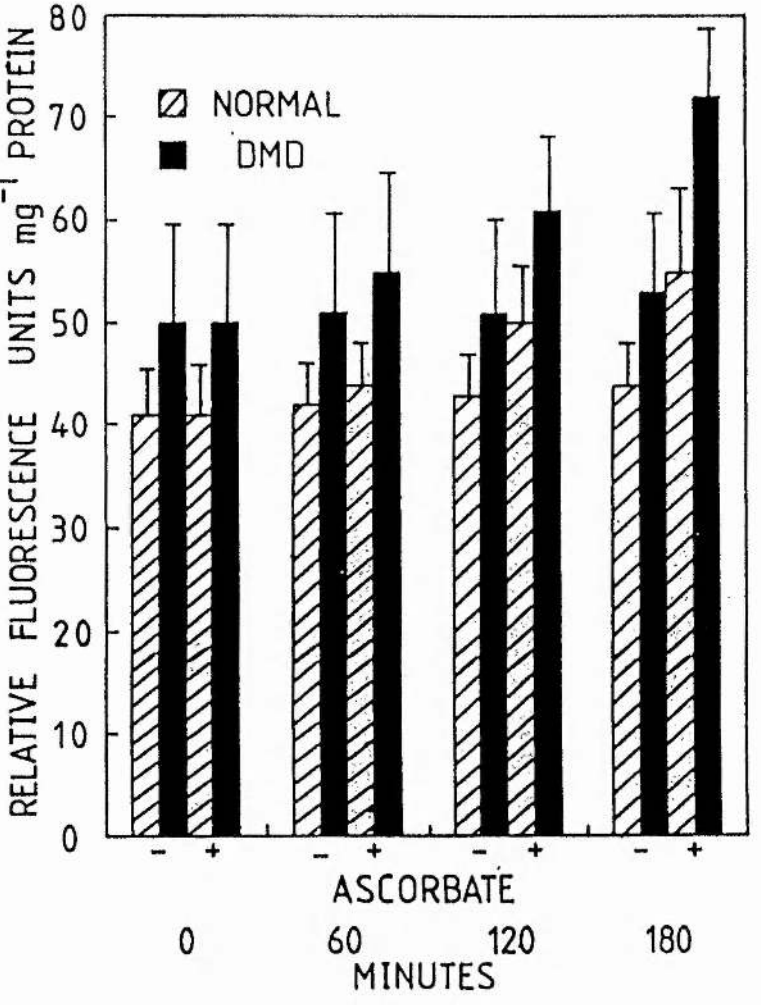


Figure 32. Fluorescent pigments produced by normal control (n=6) and DMD (n=2) whole skin fibroblast cell homogenates, expressed as relative fluorescence units/mg homogenate protein. (+) Fe^{3+}/ascorbate (12μM and 0.66mM respectively), (-) 12μM Fe^{3+}only.

3.1.4 INDUCTION AND MONITORING OF LIPID PEROXIDATION IN RAT LIVER PARTICULATE FRACTION

In order to test the antioxidant activity of the cytoplasmic fraction from CSFs it was essential to have a reliable model system for lipid peroxidation on which the inhibitory effects of our CSFs extracts could be tested.

Since the rat liver microsomal system has been extensively used by others as a model system it was decided in this work to use simply the total particulate fraction from rat liver. The experiments whose results are described in this section were carried out to determine the kinetics of enzyme-induced and nonenzyme-induced lipid peroxidation as monitored by CD, MDA and FP, in order to select a suitable single time interval at which to take samples in subsequent experiments to determine antioxidant activities.

The results for NADPH-induced lipid peroxidation are summarised in Table 10 and Figs. 33-35. As expected, NADPH induces a dramatic increase in all three indices of lipid peroxidation. The kinetics are interesting. CD and MDA both rise sharply to a maximum (9x and 7.5x control respectively) at 60 minutes after which both decline. FP on the other hand do not peak until 120 minutes (3.5x control) but remain at this maximum value for the duration of the experiment (up to 180 minutes). These observations are consistent with the known chemistry of lipid peroxidation and will be discussed more fully later.

Table 10. Lipid peroxidation products in rat liver particulate fraction induced by Fe^{3+}/ADP/NADPH (enzymic process) and Fe^{3+}/ascorbate (non-enzymic). Each value is mean of triplicate estimations ± S.D.

system / time (minutes)	CONJUGATED DIENE A_{235} mg^{-1} protein		MALONDIALDEHYDE nmol mg^{-1} protein		FLUORESCENT PIGMENT RFU mg^{-1} protein	
	ENZYMIC SYSTEM		ENZYMIC SYSTEM		ENZYMIC SYSTEM	
	-NADPH	+NADPH	-NADPH	+NADPH	-NADPH	+NADPH
0	3.83±0.24	3.83±0.24	1.83±0.16	1.83±0.16	45.8±1.5	45.8±1.5
30	4.65±0.40	27.30±1.64	1.92±0.12	7.92±0.28	54.8±3.8	118.5±5.7
60	4.70±0.00	39.13±1.16	2.75±0.24	15.70±0.91	57.8±1.5	148.5±6.2
90	5.40±0.64	33.65±2.60	3.40±0.22	15.10±0.59	58.5±1.7	198.0±4.2
120	5.18±0.84	31.18±1.56	3.43±0.16	11.73±0.24	60.0±2.4	225.8±3.8
150	4.53±0.96	25.05±2.28	2.28±0.26	11.44±0.16	62.3±1.5	223.5±6.2
180	4.10±0.80	22.30±1.44	2.12±0.75	11.60±0.13	60.8±7.1	227.3±2.9
	NON-ENZYMIC SYSTEM		NON-ENZYMIC SYSTEM		NON-ENZYMIC SYSTEM	
	-ASCORBATE	+ASCORBATE	-ASCORBATE	+ASCORBATE	-ASCORBATE	+ASCORBATE
0	3.15±0.40	3.15±0.40	3.08±0.47	3,08±0.47	34.5±3.0	34.5±3.0
30	3.55±0.40	12.20±2.08	3.46±0.34	5.90±0.49	39.0±3.5	84.0±4.9
60	3.85±0.76	32.85±1.00	3.78±0.44	13.72±0.53	36.0±0.0	100.5±5.7
90	5.40±0.92	24.60±2.08	5.26±0.86	9.94±0.50	43.5±3.0	112.5±5.7
120	3.80±0.64	17.60±1.32	3.79±0.53	9.62±1.32	42.0±3.4	118.5±10.2
150	3.55±0.76	13.30±1.44	4.75±0.56	8.59±1.96	45.0±3.5	121.5±3.0
180	3.30±0.80	13.20±1.32	4.62±1.44	6.48±0.45	45.0±3.5	118.5±3.0

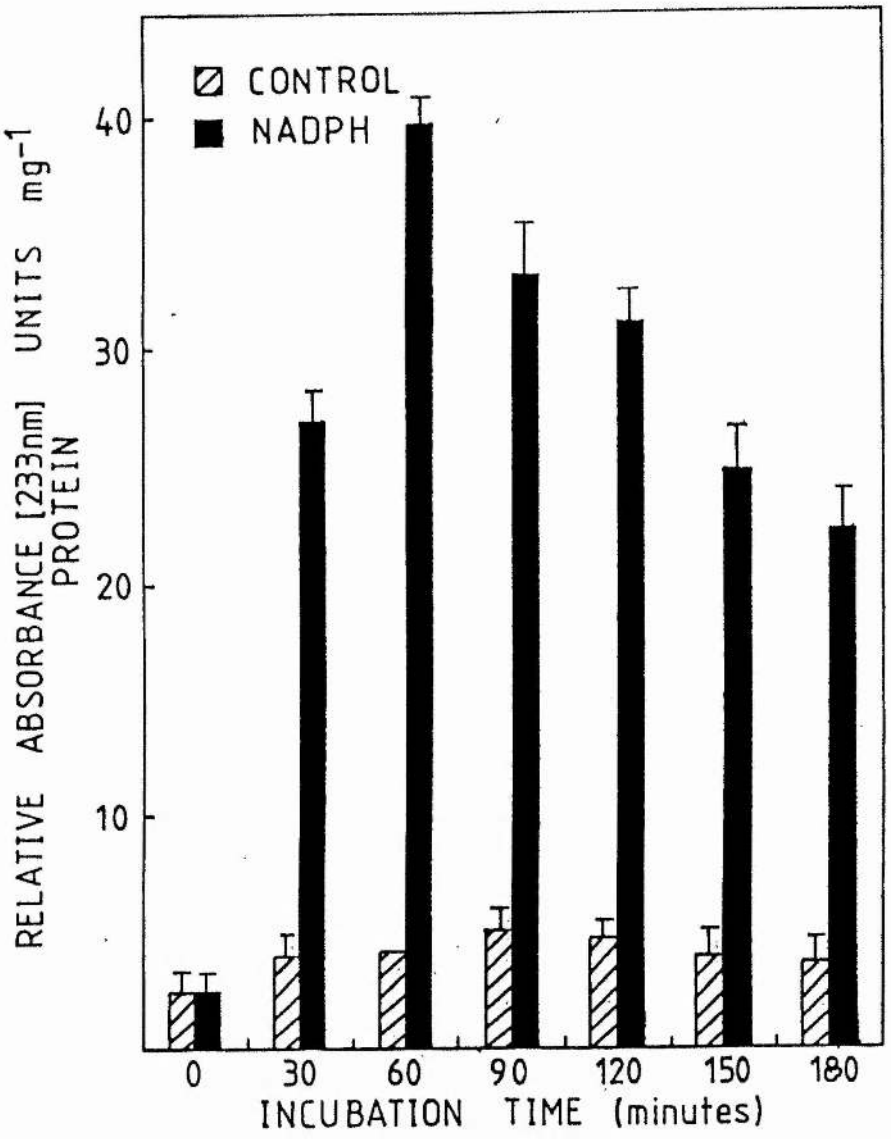


Figure 33. Effect of NADPH on liver particulate lipid peroxidation measured by conjugated diene, expressed as relative absorbance (233nm) units/mg particulate protein. Results represent average of triplicate values obtained from liver particulate without NADPH (control) and with NADPH (4mM). Error bars = standard deviation. Final concentrations in incubations:- 20mM tris-HCl, pH 7.0; 2mM ADP; 0.12mM $FeCl_3$; 20mM nicontinamide; 27mM KCl; 4mM NADPH; particulate fraction 1-3.25mg protein/ml.

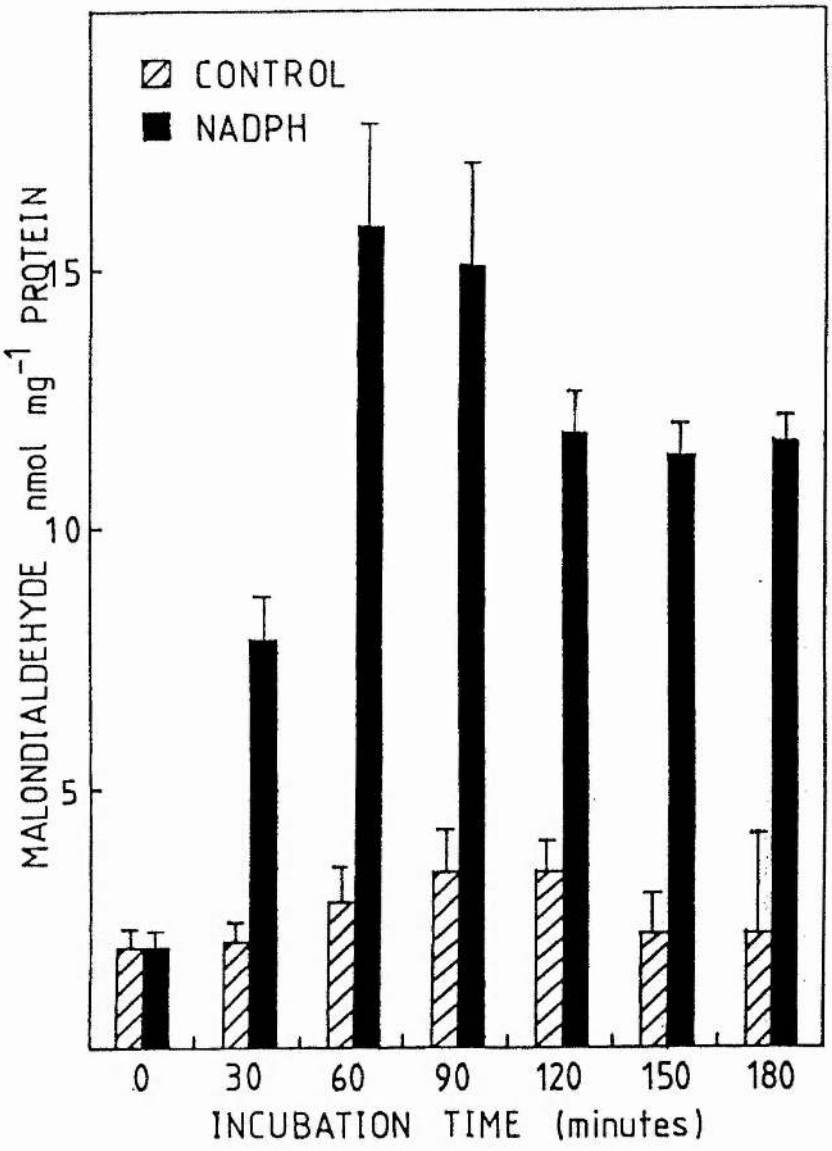


Figure 34. Effect of NADPH on liver particulate lipid peroxidation measured by thiobarbituric acid test (as MDA), expressed as nmol MDA/mg particulate protein. Results represent average of triplicate values obtained from liver particulate without NADPH (control) and with NADPH (4mM). Error bars = standard deviation. Final concentrations in incubations:- 20mM tris-HCl, pH 7.0; 2mM ADP; 0.12mM $FeCl_3$; 20mM nicontinamide; 27 mM KCl; 4mM NADPH; particulate fraction 1-3.25mg protein/ml.

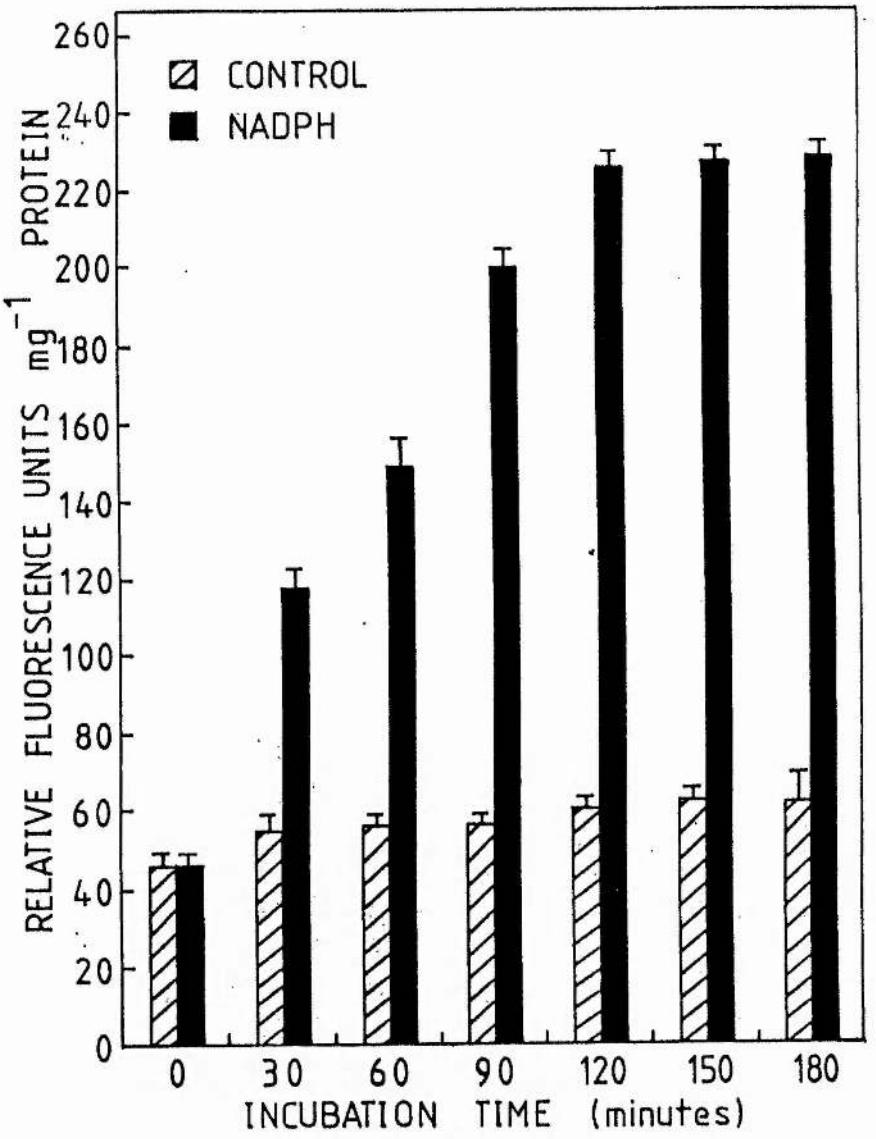


Figure 35. Effect of NADPH on liver particulate lipid peroxidation measured by fluorescent pigment , expressed as relative fluorescence units/mg particulate protein. Results represent average of triplicate values obtained from liver particulate without NADPH (control) and with NADPH (4mM). Error bars = standard deviation. Final concentrations in incubations:- 20mM tris-HCl, pH 7.0; 2mM ADP; 0.12mM $FeCl_3$ 20mM nicontinamide; 27mM KCl; 4mM NADPH; particulate fraction 1-3.25mg protein/ml.

Ascorbate/Fe^{3+} induced lipid peroxidation data are shown in Table 10 and Figs. 36-38. The kinetics are very similar to the enzymic system in that both CD and MDA reach a maximum at 60 minutes (9x and 3.5x control respectively) and decline thereafter whereas FP do not peak (3x control) until 120 minutes whereafter the levels are sustained (until 180 minutes).

On the basis of these results an incubation time of 60 minutes was chosen for future experiments since both CD and MDA are maximal at this time and FP although still increasing should be a more sensitive index and therefore still easily measureable.

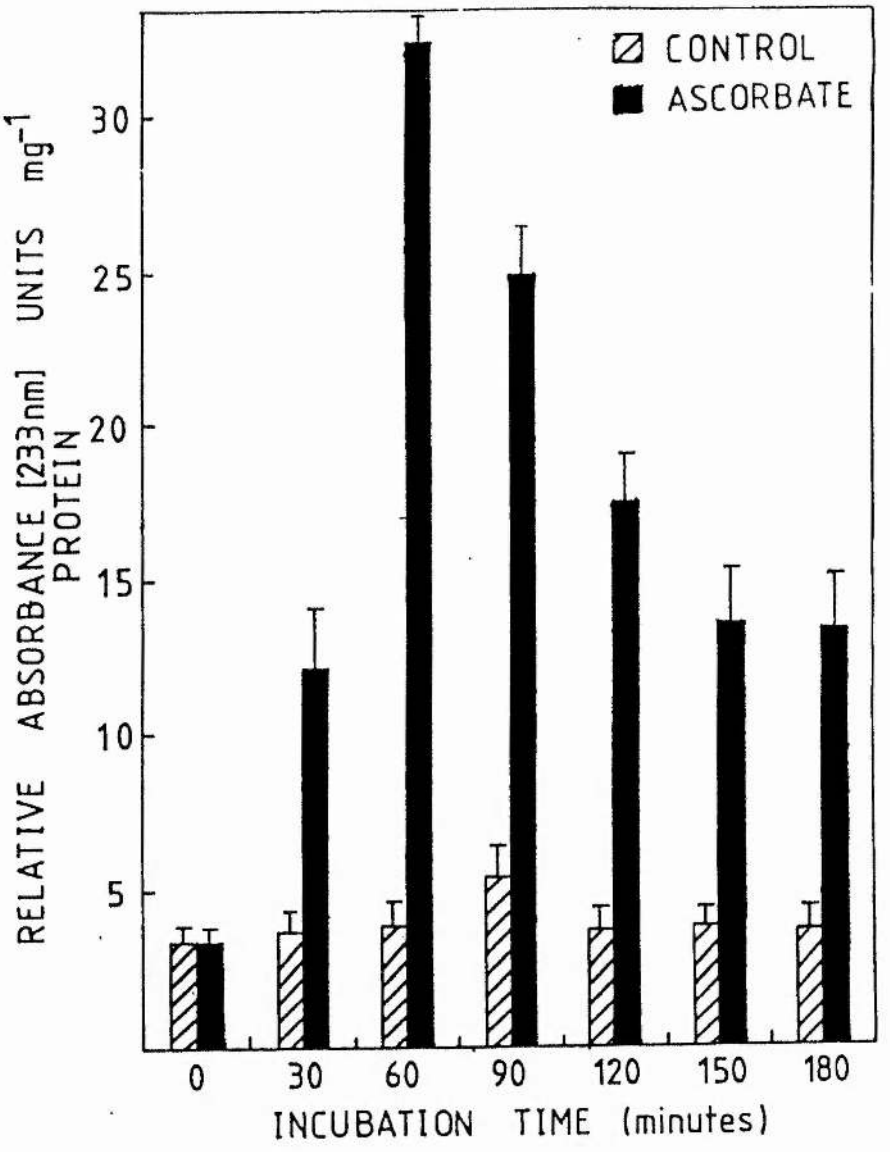


Figure 36. Effect of ascorbate on liver particulate lipid peroxidation measured by conjugated diene, expressed as relative absorbance (233nm) units/mg particulate protein. Results represent average of triplicate values obtained from liver particulate without ascorbate (control) and with ascorbate (0.66mM). Error bars = standard deviation. Final concentrations in incubations:- 10µM tris-HCl, pH 7.0; 4mM ADP; 12µM $FeCl_3$; 066mM ascorbate; particulate fraction 1.0mg protein/ml.

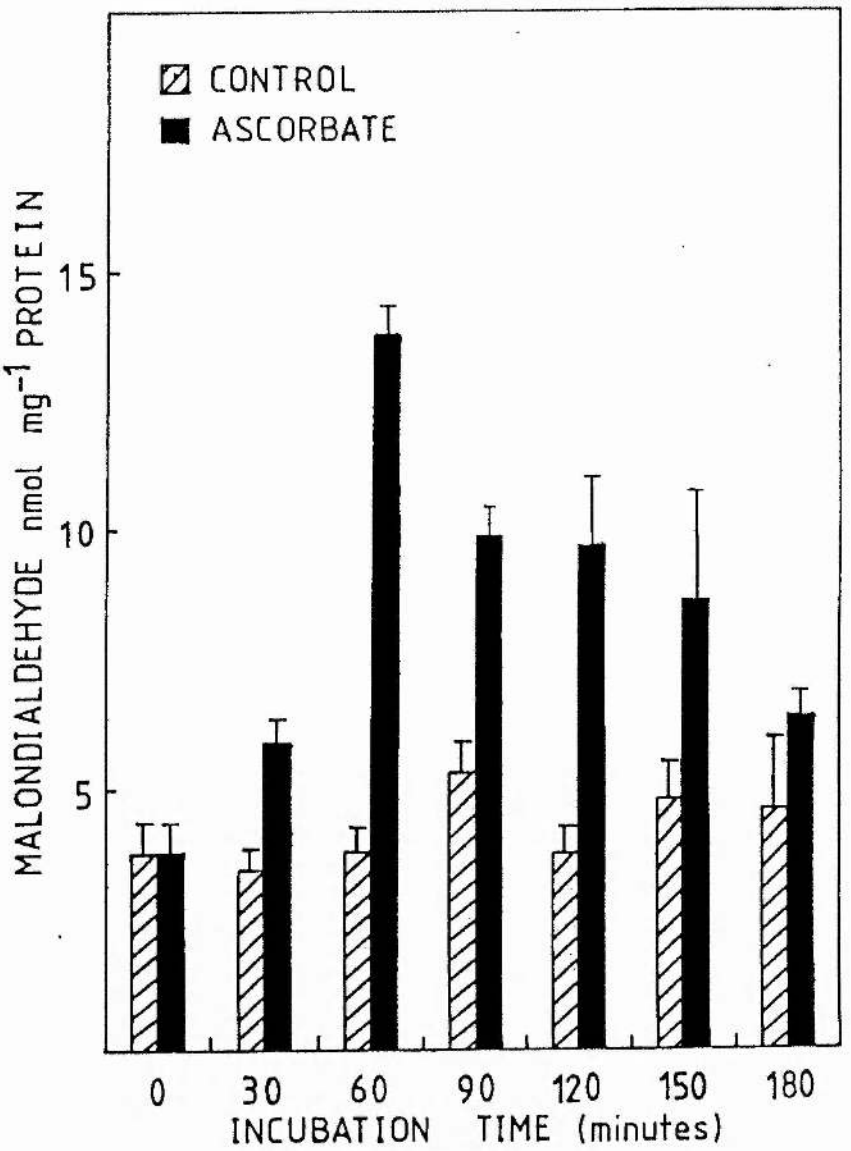


Figure 37. Effect of ascorbate on liver particulate lipid peroxidation measured by thiobarbituric acid test (as MDA), expressed as nmol MDA/mg particulate protein. Results represent average of triplicate values obtained from liver particulate without ascorbate (control) and with ascorbate (0.66mM). Error bars = standard deviation. Final concentrations in incubations:- 10μM tris-HCl, pH 7.0; 4mM ADP; 12μM $FeCl_3$; 0.66mM ascorbate; particulate fraction 1.0mg protein/mg.

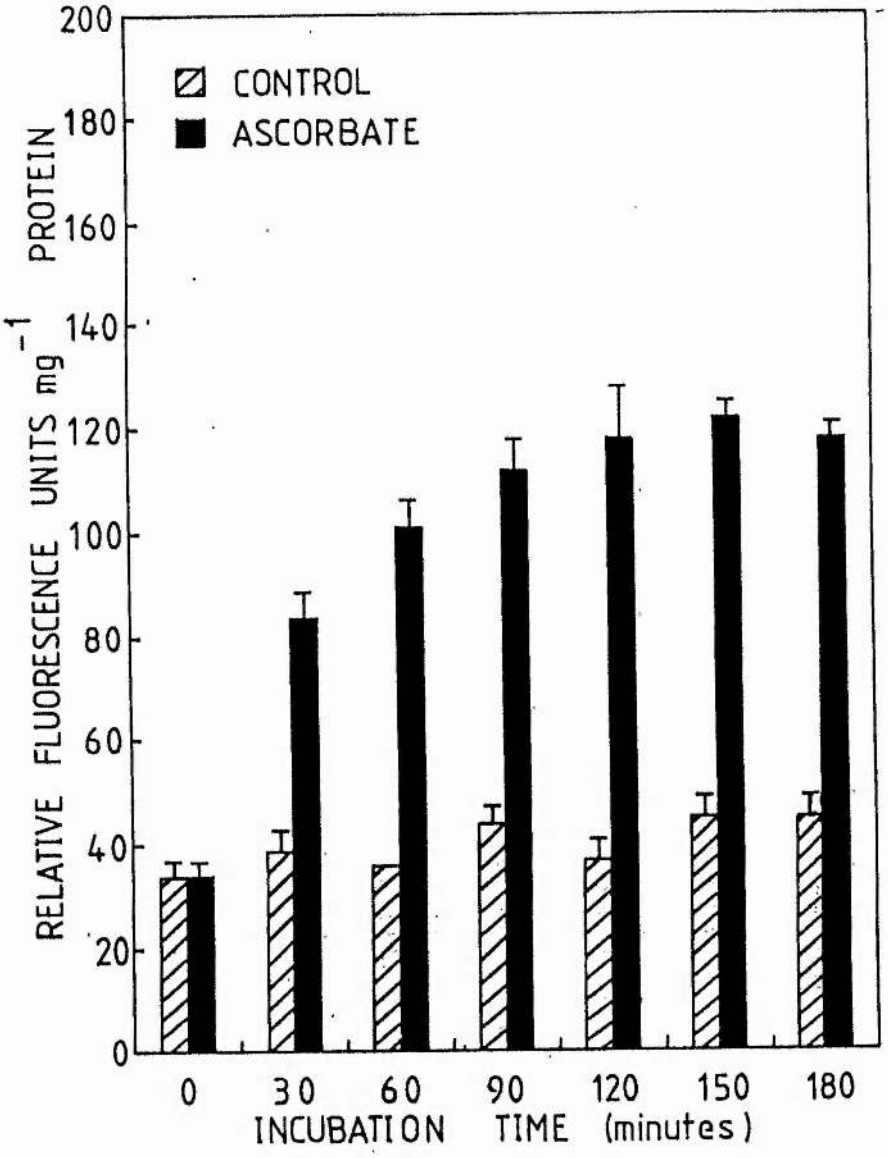


Figure 38. Effect of ascorbate on liver particulate lipid peroxidation measured by fluorescent pigment, expressed as relative fluorescence units/mg particulate protein. Results represent average of triplicate values obtained from liver particulate without ascorbate (control) and with ascorbate (0.66mM). Error bars = standard deviation. Final concentrations in incubations:- 10µM tris-HCl, pH 7.0; 4mM ADP; 12µM $FeCl_3$; 0.66mM ascorbate; particulate fraction 1.0mg protein/ml.

3.1.5 ANTIOXIDANT ACTIVITY OF CSF CYTOPLASMIC FRACTION

Previous studies in several laboratories demonstrated that a GSH-dependent enzyme, presumed at that time to be GSHPx (E.C 1.11.1.9), did not inhibit peroxidation in microsomal or mitochondrial membranes by reducing complex lipid peroxides to lipid alcohols (McCay et al.,1976). However, additional evidence indicates that another factor (but not GSHPx) from rat liver cytosol is required for the GSH-dependent suppression of lipid peroxidation. It was also found the factor is heat resistant (Gibson et al.,1980). The mechanism of this protective effect is unknown and the studies described below were carried out in an attempt to assess the total, GSH-dependent antioxidant potential of CSFs 100,000g supernatants, since it had already been shown (Hunter, personal communication) that GSHPx (and GSHR) activity was not different from normal in DMD CSFs. In this study the cytoplasmic fraction (100,000g supernatant) from CSFs from normal healthy individuals and DMD patients were tested to assess their potency in inhibition of lipid peroxidation in the rat liver particulate systems described in the foregoing section.

The results of these experiments are shown in Table 11 and Figs. 39-44. Clearly, in this experiments, cytosol from both DMD and normal control CSFs was able to suppress lipid peroxidation in the rat liver system stimulated both enzymically and non-enzymically. Although the inhibiting factor is more effective against ascorbate/Fe^{3+} rather than NADPH-induced lipid peroxidation. This effect was seen both with cytosol alone and with cytosol plus GSH.

Table 11. Lipid peroxidation products in the liver particulate system when incubated with normal control (n = 1) and DMD (n = 1) fibroblast supernatants induced by Fe^{3+}/ADP/NADPH and Fe^{3+}/ascorbate.

Each value is mean of duplicate estimation ± S.D.

Additions to Incubation Mixtures	CONJUGATED DIENES A_{235} mg^{-1} protein				MALONDIALDEHYDE nmol mg^{-1} protein				FLUORESCENT PIGMENTS Relative units mg^{-1} protein			
	Normal	% Inhibition	DMD	% Inhibition	Normal	% Inhibition	DMD	% Inhibition	Normal	% Inhibition	DMD	% Inhibition
ENZYMIC SYSTEM												
None	4.8±0.7		4.8±0.7		3.3±0.4		3.3±0.4		66±11		66±11	
NADPH	21.6±2.2		21.6±2.2		6.6±1.5		6.6±1.5		108±16		108±16	
NADPH+GSH	21.6±2.0	0	21.6±2.0	0	6.7±1.1	0	6.7±1.1	0	108±7	0	108±7	0
NADPH+ CYTOSOL*	20.1±2.3	6.9	17.5±2.3	19.0	6.5±2.6	1.5	6.2±1.5	6.1	99±16	8.3	81±16	25.0
NADPH+GSH +CYTOSOL*	20.4±5.1	5.1	13.3±5.7	38.4	6.9±2.6	(+4.5)	4.8±1.3	27.3	93±22	13.9	68±26	37.0
NON-ENZYME SYSTEM												
None	6.2±1.1		6.2±1.1		4.9±0.4		4.9±0.4		51±15		51±15	
ASCORBATE	15.2±3.3		15.2±3.3		14.0±2.3		14.0±2.3		203±20		203±20	
ASCORBATE+ GSH	14.7±2.2	3.3	14.7±2.2	3.3	14.9±2.9	(+6.4)	14.9±2.9	(+6.4)	151±15	25.6	151±15	25.6
ASCORBATE +CYTOSOL*	14.6±1.8	3.9	12.5±2.6	17.8	14.6±2.2	(+4.3)	13.8±2.2	1.4	144±20	29.1	119±29	41.4
ASCORBATE +GSH+ CYTOSOL*	14.7±5.8	3.3	10.3±6.6	32.2	14.6±2.6	(+4.3)	12.7±3.3	9.3	125±15	38.4	107±22	47.3

* Cytosol protein/particulate protein = 3.38:1

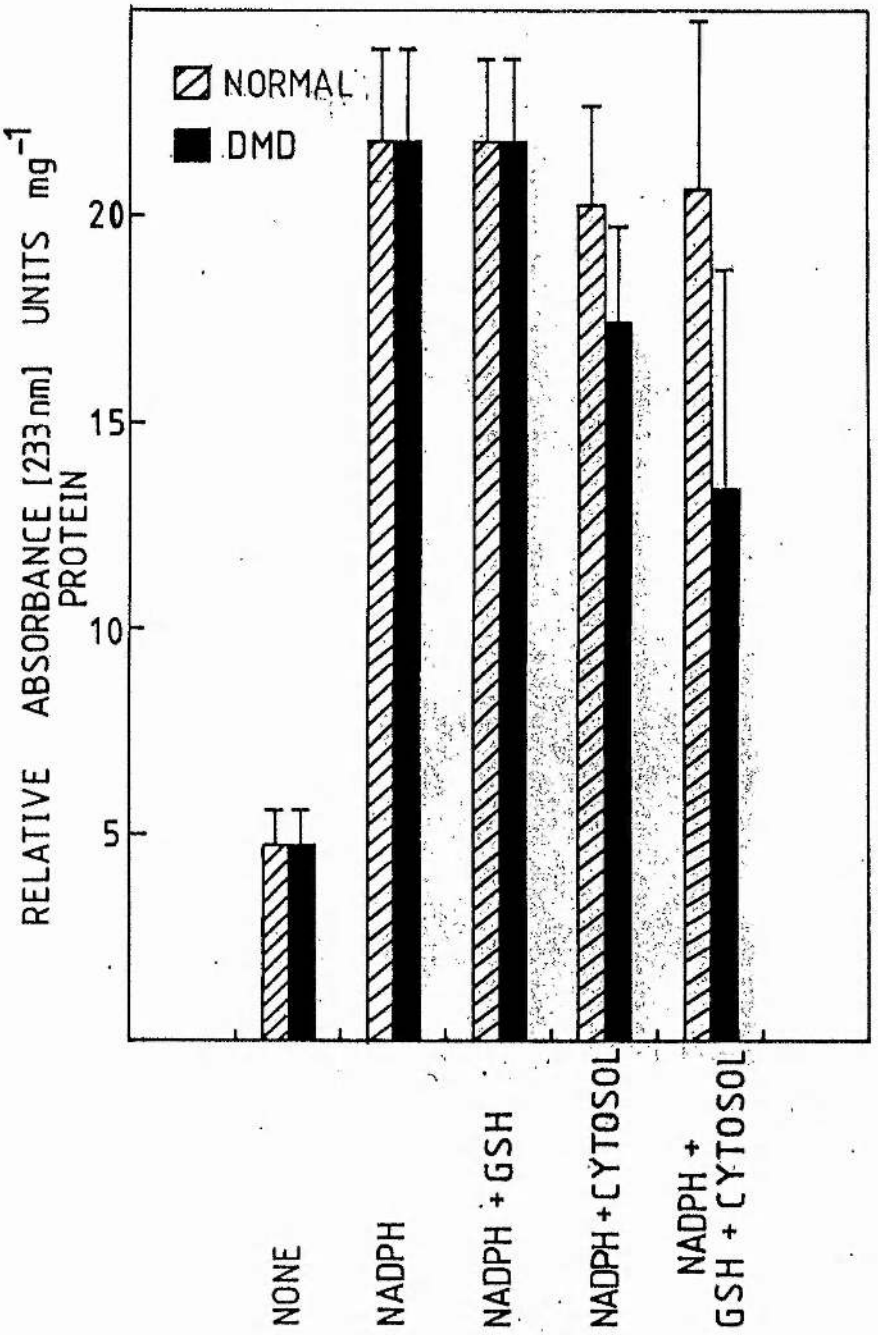


Figure 39. Lipid peroxidation products (conjugated diene) after 60 mins incubation of liver particulate fraction with (a) no addition; (b) NADPH (4mM); (c) NADPH (4mM) + glutathione (10mM); (d) NADPH (4mM) + cytosol (3.38mg protein/mg particulate protein); (e) NADPH (4mM) + glutathione (10mM) + cytosol (3.38mg protein/mg particulate protein).

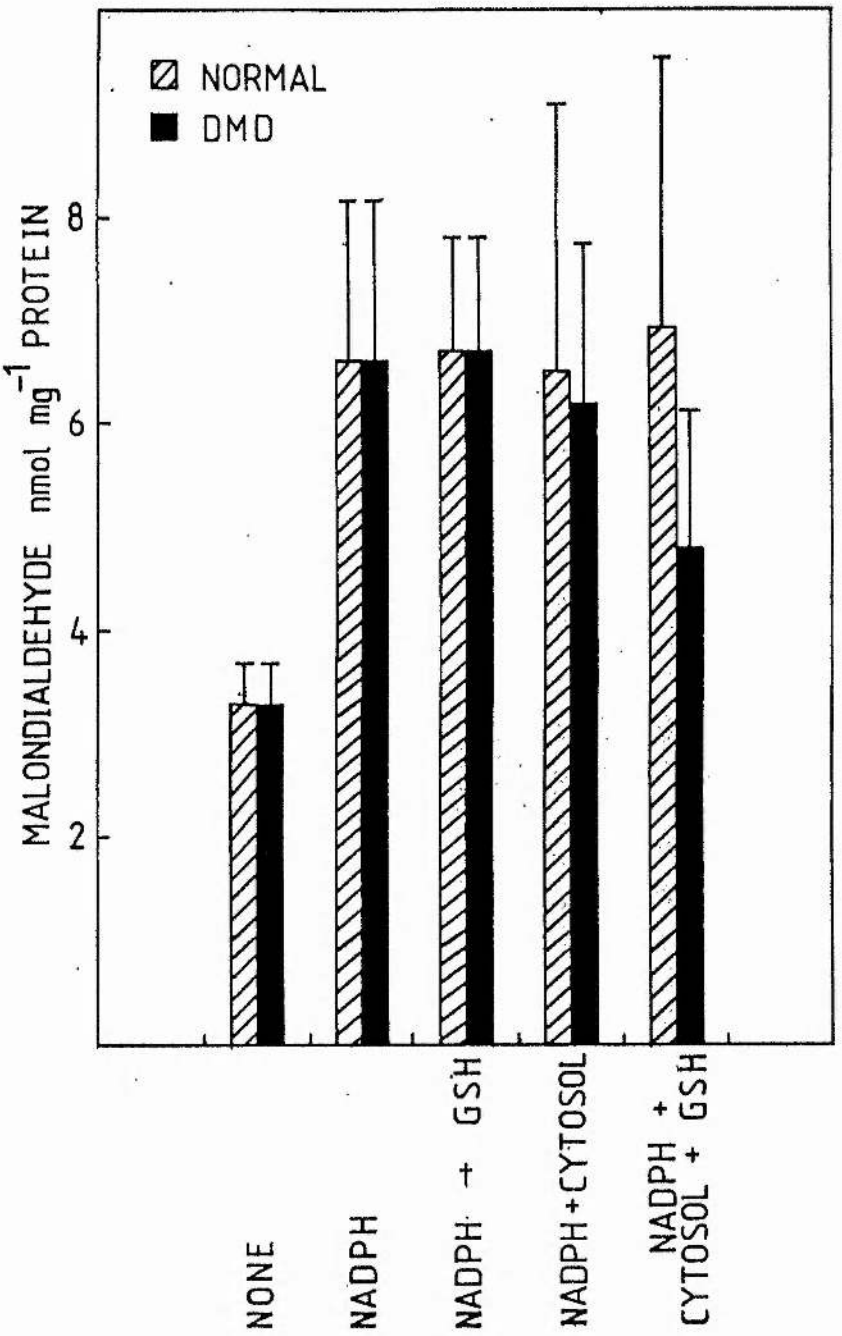


Figure 40. Lipid peroxidation products (malondialdehyde) after 60 mins incubation of liver particulate fraction with (a) no addition; (b) NADPH (4mM); (c) NADPH (4mM) + glutathione (10mM); (d) NADPH (4mM) + cytosol (3.38mg protein/mg particulate); protein); (e) NADPH (4mM) + glutathione (10mM) + cytosol (3.38mg protein/mg particulate protein).

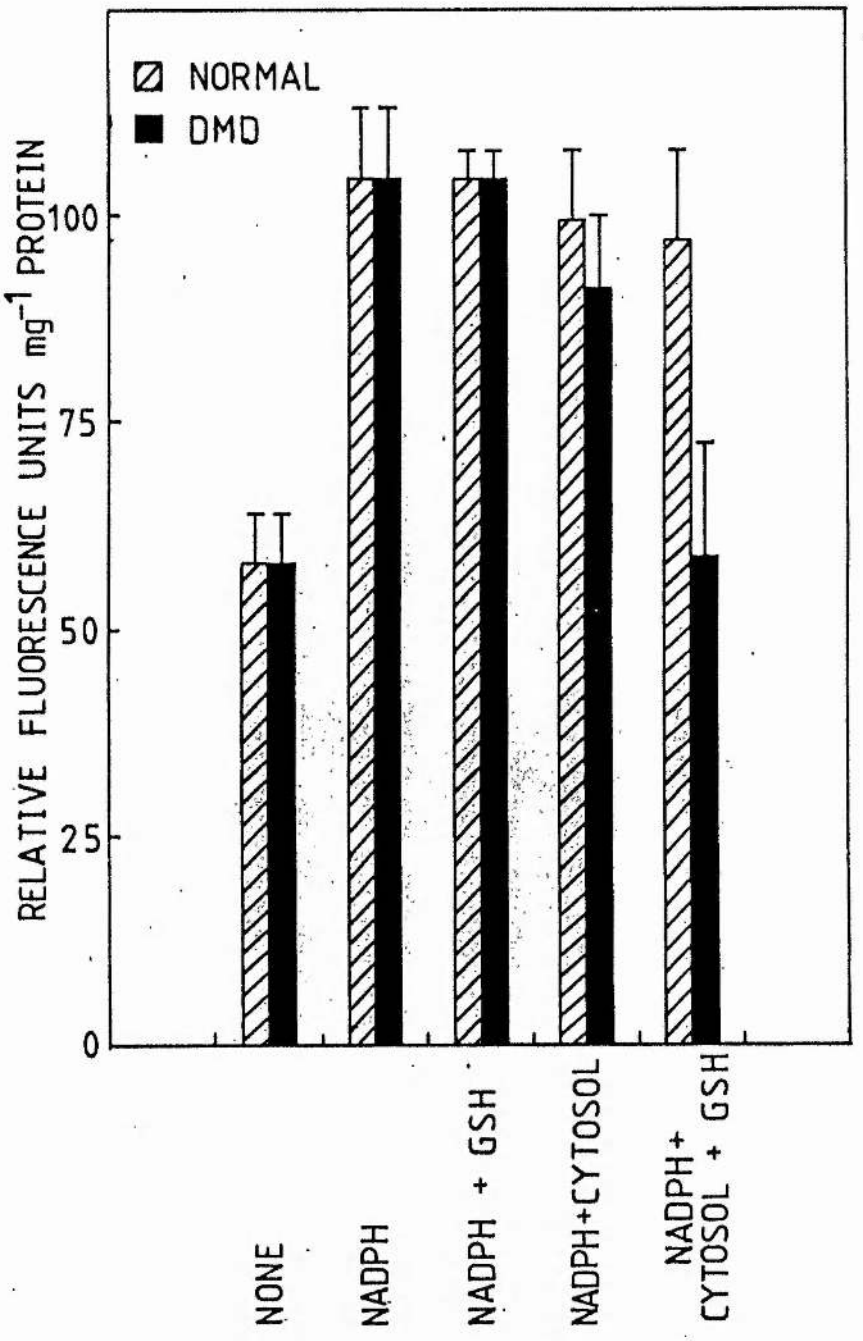


Figure 41. Lipid peroxidation products (fluorescent pigments) after 60 mins incubation of liver particulate fraction with (a) no addition; (b) NADPH (4mM); (c) NADPH (4mM) + glutathione (10mM); (d) NADPH (4mM) + cytosol (3.38mg protein/mg particulate protein); (e) NADPH (4mM) + glutathione (10mM) + cytosol (3.38mg protein/mg particulate protein).

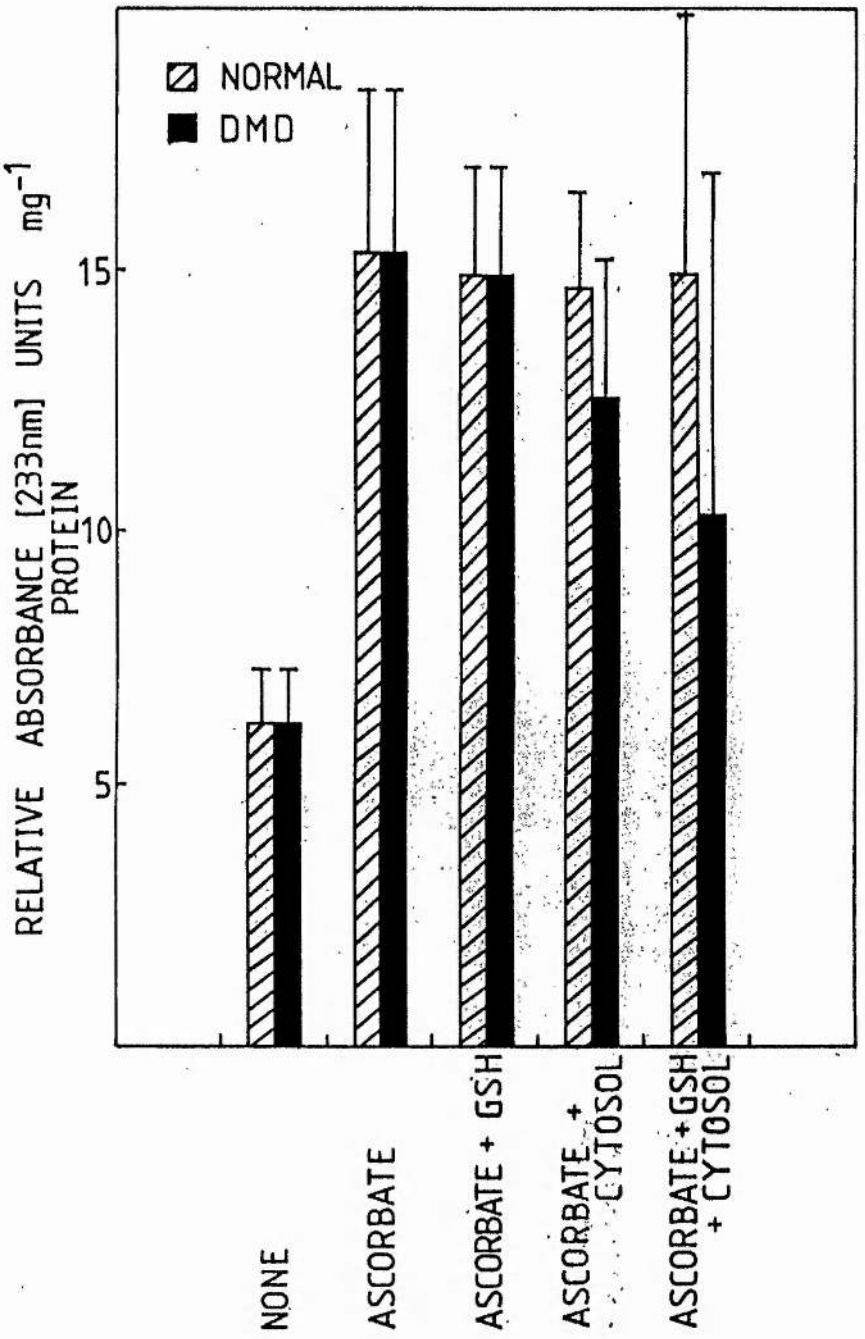


Figure 42. Lipid peroxidation products (conjugated diene) after 60 mins incubation of liver particulate fraction with (a) no addition; (b) ascorbate (0.66mM); (c) ascorbate (0.66mM) + glutathione (10mM); (d) ascorbate (0.66mM) + cytosol (3.38mg protein/mg particulate protein); (e) ascorbate (0.66mM) + glutathione (10mM) + cytosol (3.38mg protein/mg particulate protein).

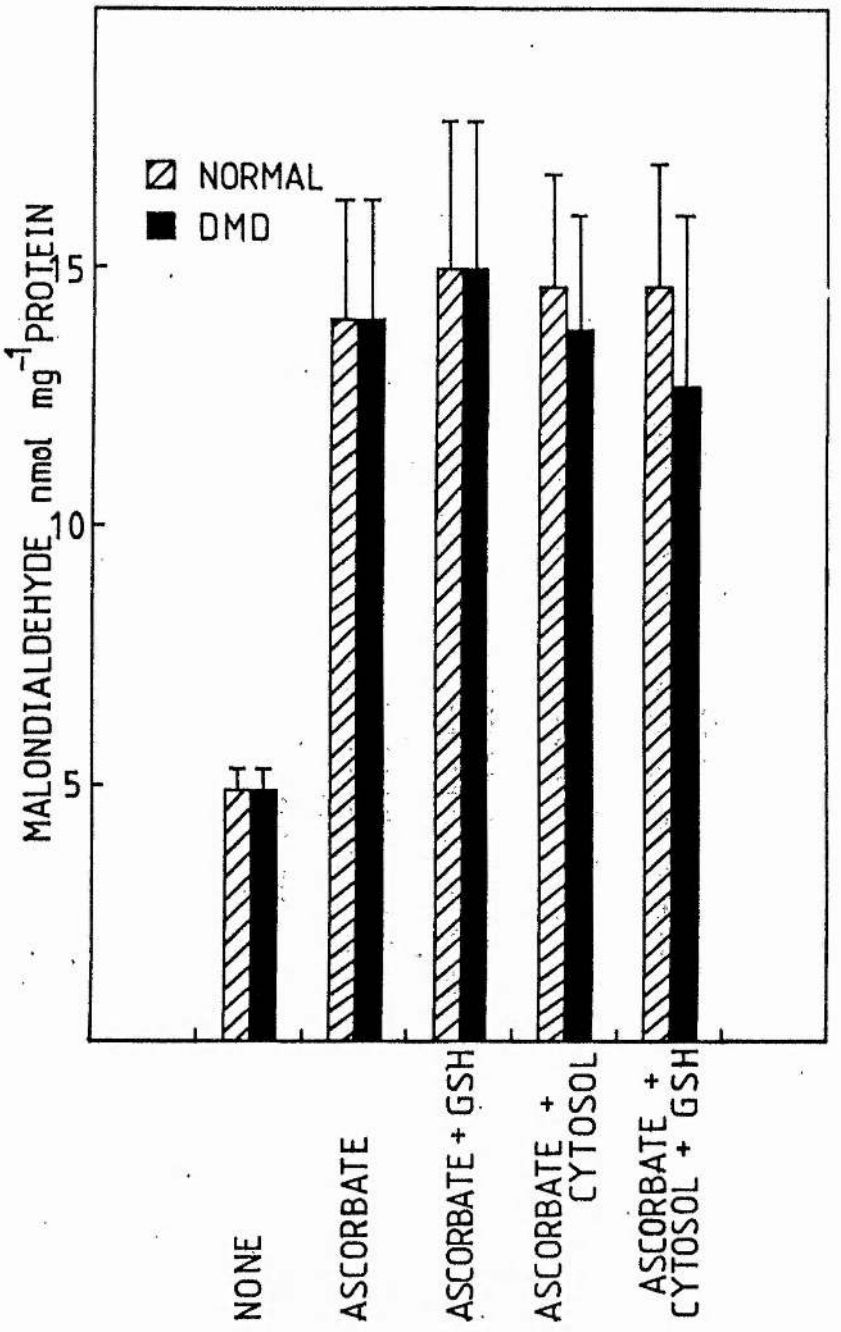


Figure 43. Lipid peroxidation products (malondialdehyde) after 60 mins incubation of liver particulate fraction with (a) no addition; (b) ascorbate (0.66mM); (c) ascorbate (0.66mM) + glutathione (10mM); (d) ascorbate (0.66mM) + cytosol (3.38mg protein/mg particulate protein); (e) ascorbate (0.66mM) + glutathione (10mM) + cytosol (3.38mg protein/mg particulate protein).

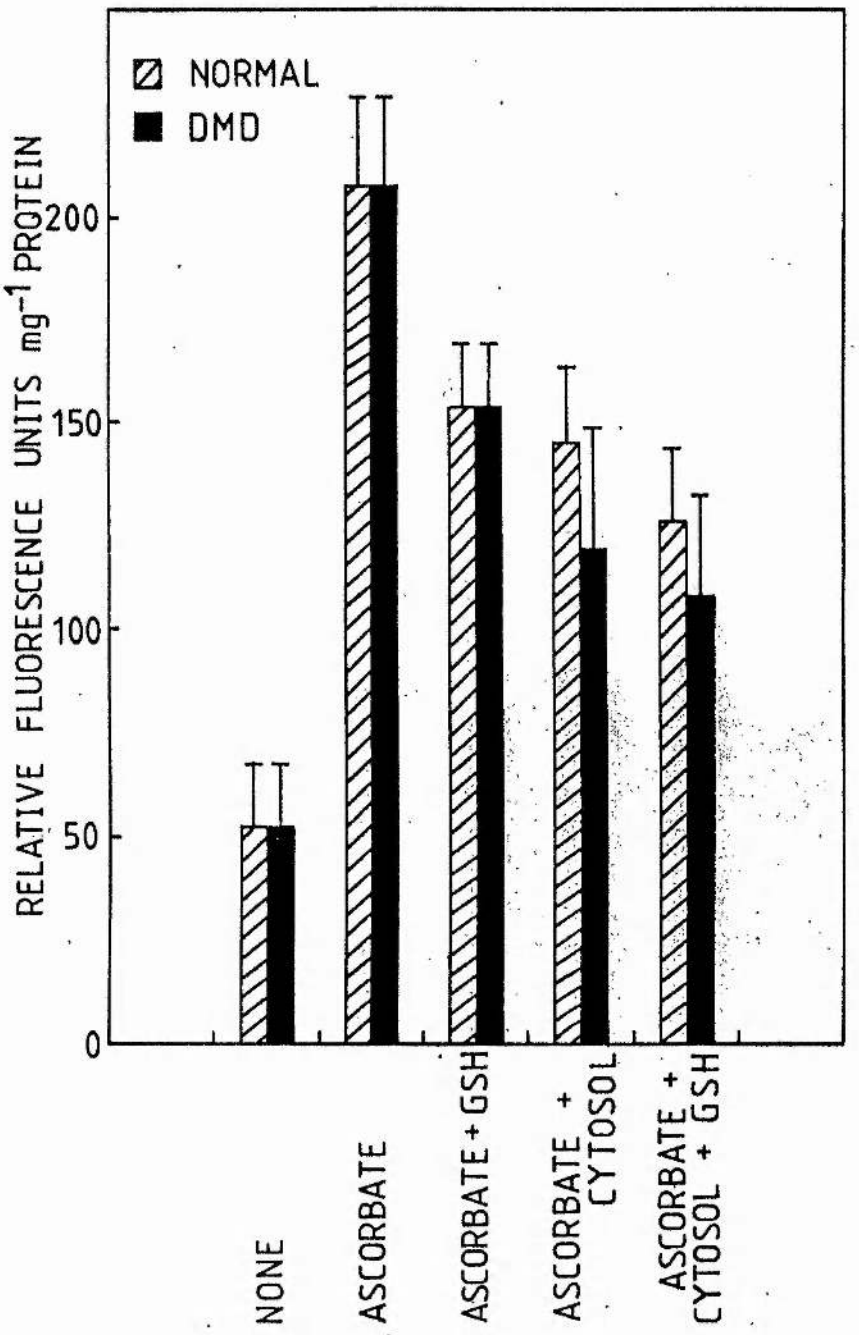


Figure 44. Lipid peroxidation products (fluorescent pigments) after 60 mins incubation of liver particulate fraction with (a) no addition; (b) ascorbate (0.66mM); (c) ascorbate (0.66mM) + glutathione (10mM); (d) ascorbate (0.66mM) + cytosol (3.38mg protein/mg particulate protein); (e) ascorbate (0.66mM) + glutathione (10mM) + cytosol (3.38mg protein/mg particulate protein).

Two important points can be made about these results:-

(i) whereas with DMD supernatant, the addition of GSH+cytosol produced increased inhibition under most conditions, there was little difference between the inhibition obtained with control cytosol alone and with added GSH;

(ii) although perhaps not statistically significant due to the small number of samples tested, nevertheless there is a convincing and consistent trend under all experimental conditions for the DMD cytosol to be more effective as an antioxidant than control. The significance of these results will be dealt with in the discussion.

3.2 PLASMA STUDIES

3.2.1 PLASMA LIPID PEROXIDATION PRODUCTS

3.2.1.1 FRESH SAMPLES

The plasma lipid peroxidation products in DMD measured by u.v absorbance (CD), TBA test (MDA) and FP were significantly higher than in a group of normal boys matched for age (Table 12). One carrier's plasma was also examined but showed no significant difference from normal control plasma. These results are summarised in Figs. 45-47. Levels of CD were raised by 77% ($P<0.02$), TBA reactive materials by 35% ($P<0.01$) and FP by 70% ($P<0.001$). Possible correlations between lipid peroxidation products (CD, MDA and FP) in DMD and normal control plasma were calculated. The results are shown in Table 13.

A correlation coefficient of 0.73 was found between DMD MDA and FP indicating that they were significantly positively correlated ($P<0.02$). Otherwise no significant correlation between any of the indices for normal or DMD plasma was found.

Table 12. Lipid peroxidation products in human plasma from patients with DMD and age-related normal control subjects.

NORMAL CONTROLS (CHILDREN)

Normal Plasma	Malondialdehyde nmol ml^{-1} plasma	Conjugated Diene A_{235} ml^{-1} plasma	Fluorescent Pigment RFU ml^{-1} Plasma
PA	4.43±0.49	1.31±0.30	11.4 ± 0.6
A	4.88±0.35	1.17±0.12	11.0 ± 0.4
LH	2.96±0.38	1.29±0.05	12.3 ± 0.4
MG	4.18±0.28	1.16±0.07	10.0 ± 1.2
GP	3.12±0.24	1.16±0.16	10.6 ± 0.6
HT	2.84±0.61	1.14±0.16	12.1 ± 0.5
AE	2.92±0.48	1.14±0.24	11.1 ± 0.0
FF	4.64±0.42	1.40±0.44	11.1 ± 1.2
GR	4.56±0.84	1.42±0.42	8.8 ± 0.5
EP	3.24±0.61	1.28±0.24	11.3 ± 0.3
n = 10	3.77 ± 0.82	1.24 ± 0.11	10.9 ± 0.9

Age between 4 and 16

Table 12 (Continued)

NORMAL CONTROLS (ADULTS)

Normal Plasma	Conjugated Diene A_{235} ml^{-1} plasma	Malondialdehyde nmol ml^{-1} plasma	Fluorescent Pigment RFU ml^{-1} Plasma
EJ	1.92±0.40	4.81±0.50	12.2±0.8
JM	1.75±0.40	4.43±0.49	15.4±1.2
LC	1.76±0.30	5.12±0.47	11.0±0.8
AG	1.31±0.30	4.88±0.35	10.3±1.0
WML	1.25±0.11	4.66±0.42	11.0±0.6
CRS	1.17±0.12	5.45±0.52	13.5±1.5
JDB	1.17±0.12	4.10±0.27	9.3±2.0
ML1	1.29±0.05	4.63±0.36	12.3±1.9
ML2	1.16±0.07	5.19±0.26	15.2±2.6
DK	1.24±0.05	2.93±0.30	13.0±1.5
MISH	1.20±0.07	4.10±0.20	12.4±1.6
n = 11	1.38±0.28	4.57±0.69	12.3 ± 1.8

Age between 20 and 37

Table 12 (Continued)

DUCHENNE MUSCULAR DYSTROPHY

Duchenne Plasma	Conjugated Diene A_{235} ml^{-1} plasma	Malondialdehyde nmol ml^{-1} plasma	Fluorescent Pigment RFU ml^{-1} plasma
SO	2.13±0.30	5.03±0.22	23.3 ± 0.8
NRJ	2.27±0.40	6.44±0.19	22.0 ± 0.8
DC	1.75±0.40	5.47±0.14	23.1 ± 0.4
JT	1.79±0.60	5.01±0.17	19.0 ± 5.4
DT	4.93±0.10	4.47±0.24	15.4 ± 1.0
MF	2.40±0.60	6.28±0.13	23.0 ± 0.5
DR	1.46±0.64	4.47±0.24	18.0 ± 1.2
JM	1.79±0.60	5.12±0.12	12.9 ± 0.3
PM	1.39±0.41	3.64±0.43	12.5 ± 0.2
n = 9	2.21 ± 1.01	5.10 ± 0.88	18.7 ± 4.3

Age between 3 and 16

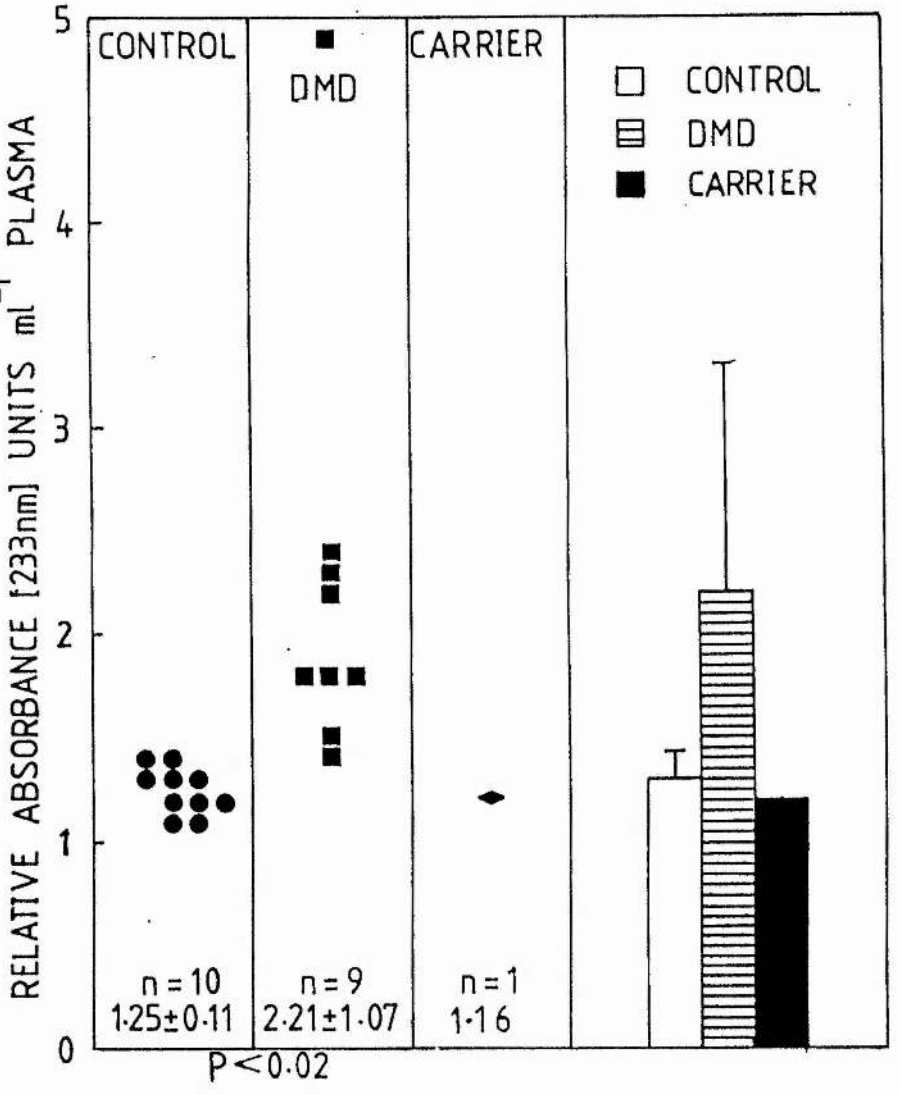


Figure 45. Distribution of conjugated diene concentration in plasma of patients with DMD (■), a carrier (◆) and normal controls (●). Each point represents the mean of duplicate assays on an individual patient, carrier or control.

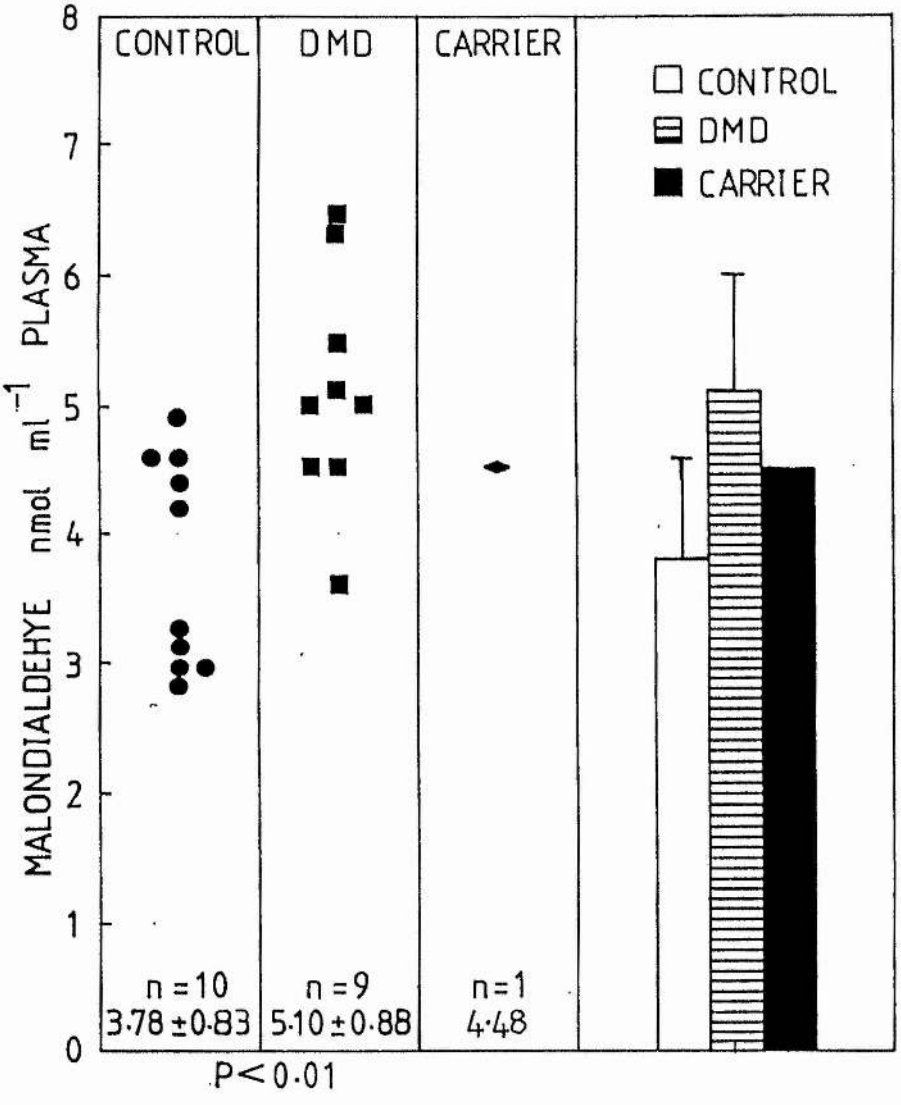


Figure 46. Distribution of TBA-reactive material concentration in plasma of patients with DMD (■), a carrier (◆) and normal controls (●). Each point represents the mean of duplicate assays on an individual patient, carrier or control..

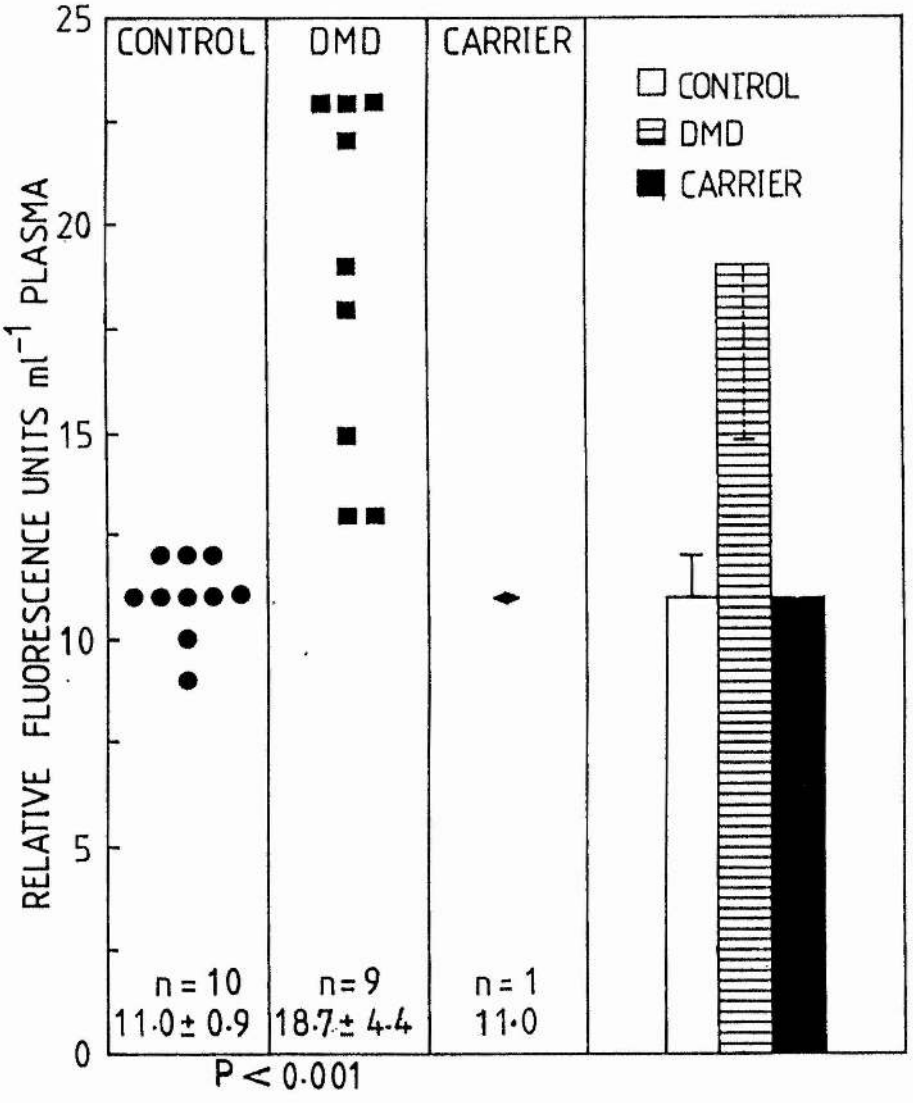


Figure 47. Distribution of fluorescent pigment concentration in plasma of patients with DMD (■), a carrier (◆) and normal controls (●). Each point represents the mean of duplicate assays on an individual patient, carrier or control.

Table 13. Correlation coefficients for pairs of plasma lipid peroxidation products.

INDICES COMPARED	CONJUGATED DIENE + MALONDIALDEHYDE		CONJUGATED DIENE + FLUORESCENT PIGMENT		MALONDIALDEHYDE + FLUORESCENT PIGMENT	
Samples	Normal (n=10)	Duchenne (n=9)	Normal (n=10)	Duchenne (n=9)	Normal (n=10)	Duchenne (n=9)
Correlation Coefficients (r)	0.481	0.016	-0.345	-0.093	-0.553	0.729
Probability (P)	NS	NS	NS	NS	NS	P<0.02*

NS = Not Significant

* = Significant Correlation

In this study plasma lipid peroxidation products were estimated in normal adults and children plasma samples. As shown in Table 14 and Figs. 48-50, the products revealed no significant difference except for MDA which was increased 20% in plasma from adults than children (Fig. 49; Table 14; $P<0.05$).

Table 14. Comparison of plasma lipid peroxidation products in normal children and adults with DMD patients.

TYPE OF SAMPLES	CONJUGATED DIENE A_{235} ml^{-1} plasma (MEAN ± S.D.)	MALONDIALDEHYDE nmol ml^{-1} plasma (MEAN ± S.D.)	FLUORESCENT PIGMENT RFU ml^{-1} plasma (MEAN ± S.D.)
NORMAL ADULTS (n = 11)	1.38 ± 0.28	4.57 ± 0.69	12.1 ± 1.8
NORMAL CHILDREN (n = 10)	1.24 ± 0.11	3.77 ± 0.82	10.9 ± 0.9
DUCHENNE (n = 9)	2.21 ± 1.01	5.10 ± 0.88	18.7 ± 4.3

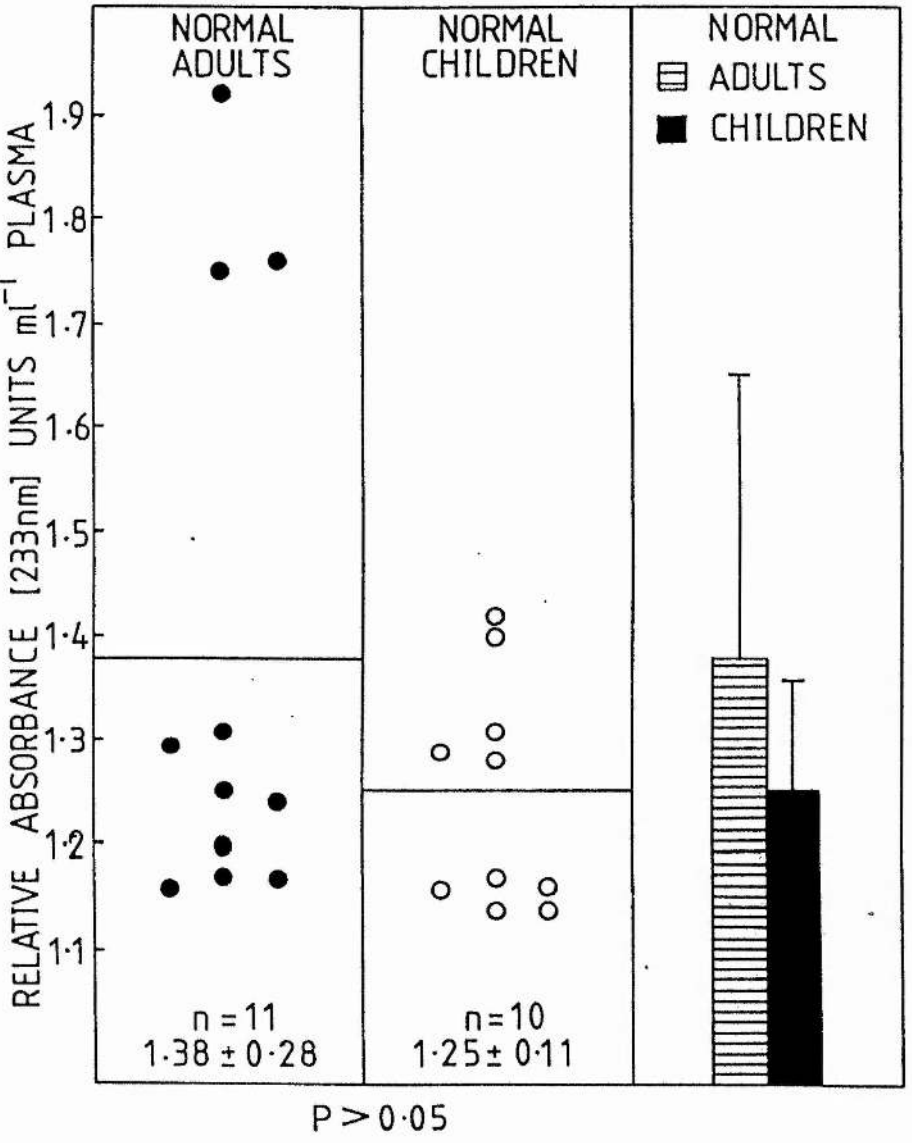


Figure 48. Distribution of conjugated diene concentration in plasma of normal adults (●) and children (○). Each point represents the mean of duplicate assays on an individual.

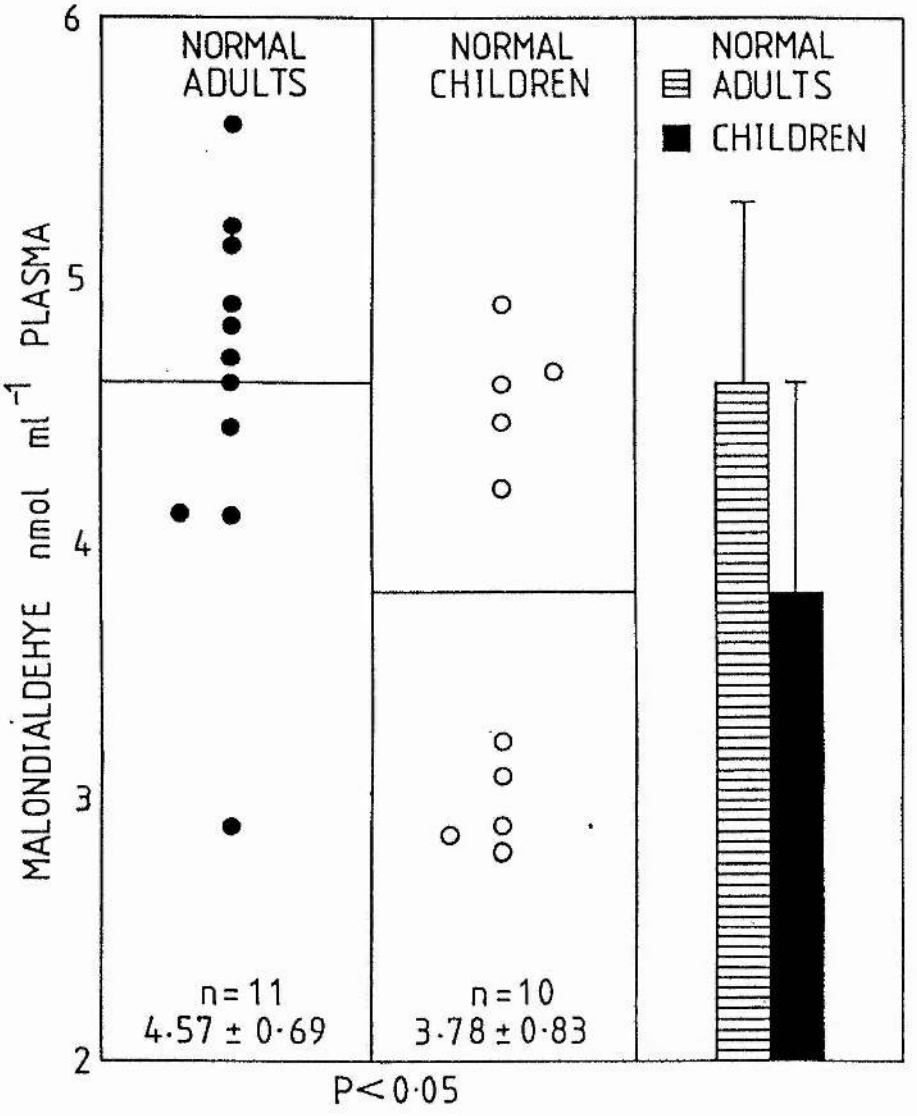


Figure 49. Distribution of malondialdehyde concentration in plasma of normal adults (●) and children (○). Each point represents the mean of duplicate assays on an individual.

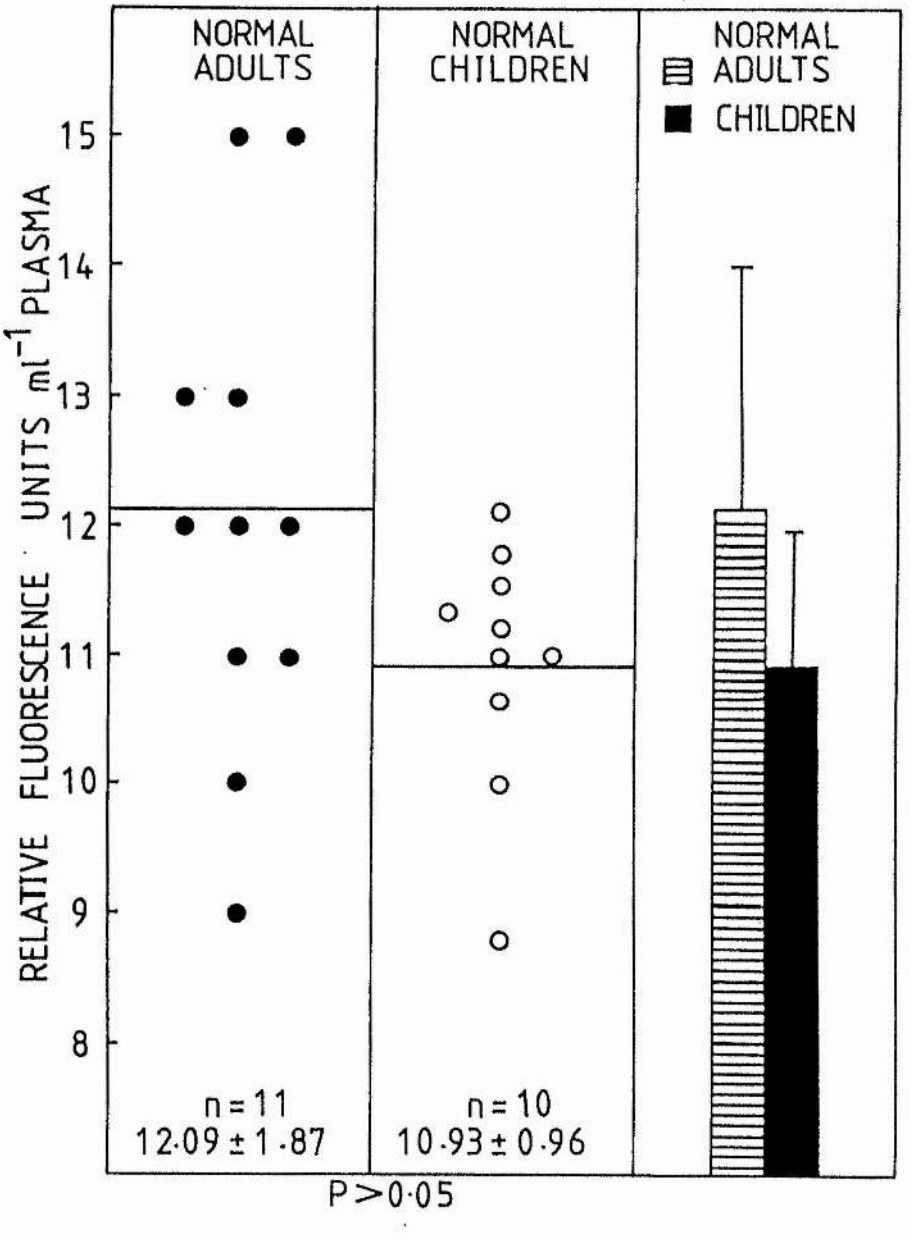


Figure 50. Distribution of fluorescent pigments concentration in plasma of normal adults (●) and children (○). Each point represents the mean of duplicate assays on an individual.

3.2.1.2 EFFECT OF STORAGE ON LIPID PEROXIDATION PRODUCTS

Because it is difficult with a relatively rare disease like DMD to arrange for simultaneous collection of several samples, it can be advantageous to accumulate samples over a period of months (if not years) prior to biochemical testing. An important pre-requisite, of course is that the parameter being measured does not alter with storage. During the course of this work, analyses were performed on samples which had been stored at -20°C for varying periods of time to determine the effects of storage. The majority of the data is for samples from different individuals but some results refer to samples from the same individual (as indicated below). Table 15 shows data for stored samples from different individuals.

The CD concentration (A_{235}) appears not to vary significantly with storage time for either control or DMD samples where both MDA and FP increase significantly after storage (Fig. 51). For both controls and DMD samples MDA increases approximately twofold whereas FP increase approximately three fold, after storage for 3 years. The kinetics of the changes deserve comment. Fig. 52 shows that whereas in DMD plasma, the MDA concentration increases almost linearly up to two years, falling off thereafter, for normal plasma there is a lag periods of a year before MDA increases appreciably. Fig. 53 shows that in both DMD and normal plasma FP increase at a similar rate, tailing off after two years.

Table 15. Lipid peroxidation products in stored plasma (-20°C) from patients with DMD and normal control subjects at various times (years). Number of samples for different storage period are as follow: 0 (N*=11, D**=9), 0.5 (N=6, D=4), 1.0 (N=6, D=2), 2.0 (N=4, D=3) and 3.0 (N=5, D=3). Each value represents the mean for each group of subjects and assay were carried out in duplicate estimations ± S.D.

Storage period (years)	CONJUGATED DIENE A_{235} ml^{-1} plasma		MALONDIALDEHYDE nmol ml^{-1} plasma		FLUORESCENT PIGMENT RFU ml^{-1} plasma	
	NORMAL	DUCHENNE	NORMAL	DUCHENNE	NORMAL	DUCHENNE
0	1.20±0.28	2.21±1.01	4.57±0.69	5.10±0.88	12.0 ± 1.8	18.7 ± 4.3
0.5	1.21±0.41	2.20±1.13	4.74±0.74	6.32±2.54	21.1 ± 2.1	32.0 ± 6.4
1.0	1.22±0.38	2.15±0.94	5.25±2.22	9.08±2.69	32.1 ± 14.5	47.7 ± 11.4
2.0	1.21±0.46	2.13±0.93	8.45±2.46	11.0±2.80	41.7 ± 13.0	56.0 ± 3.1
3.0	1.20±0.54	2.12±1.41	9.00±1.60	11.30±2.97	42.6 ± 3.5	60.1 ± 5.9

*N = Normal control, **D = Duchenne muscular dystrophy.

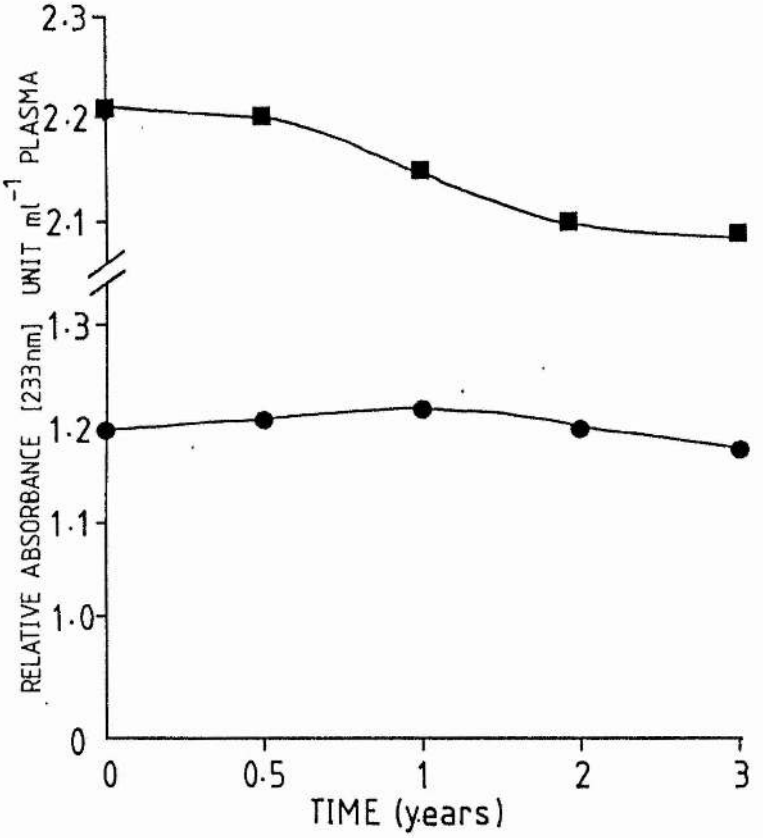


Figure 51. Conjugated diene concentration in plasma at various storage (-20°C) times, expressed as relative absorbance (233nm) units/ml plasma. Each point represents the mean for each group of subjects and assay were carried out in duplicate estimations.

●——●, control.

■——■, DMD.

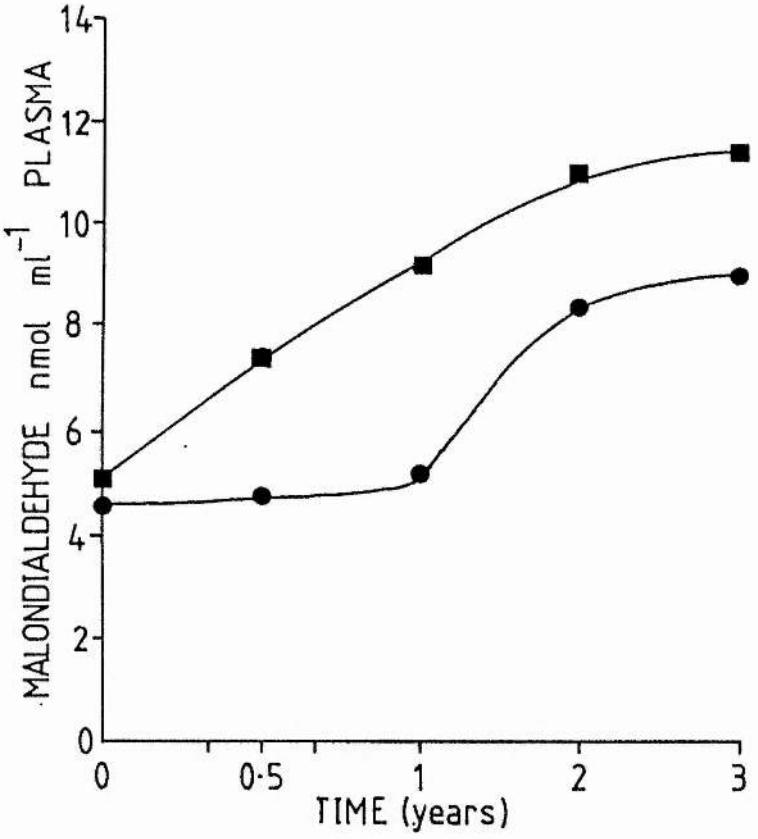


Figure 52. Malondialdehyde concentration in plasma at various storage (-20°C) times, expressed as nmol/ml plasma. Each point represents the mean for each group of subjects and assay were carried out in duplicate estimations.

●—●, control.

■—■, DMD.

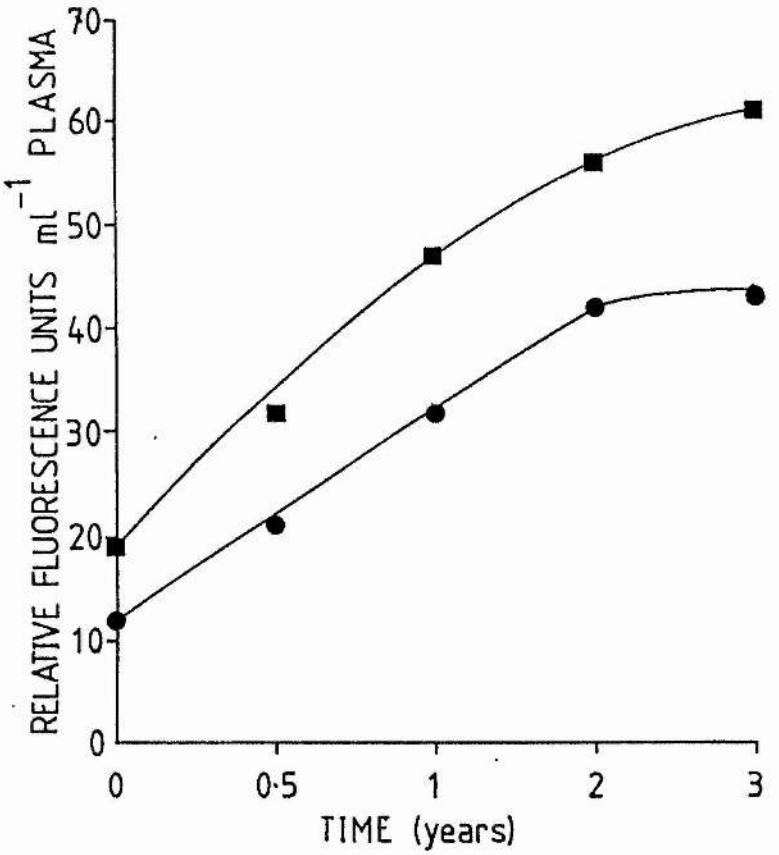


Figure 53. Fluorescent pigment concentration in plasma at various storage (-20°C) times, expressed as relative fluorescence units/ml plasma. Each point represents the mean for each group of subjects and assay were carried out in duplicate estimations.

●——●, control.

■——■, DMD.

Since most samples would probably not be stored for longer than a few months at most and since the shortest storage time investigated in the preceding study was 6 months, a further experiment was carried out assaying lipid peroxidation products from two pools of plasma for 2 normal adult individuals (A+B) at monthly intervals for 6 months. These results are shown in Table 16 and Fig. 54. There is a significant increase in both MDA (12% A and B) and FP (42%A; 64% B) on storage for 6 months but it is important to note that for both samples there was no increase in either parameter over the first month but that after this there is a linear increase up to 6 months.

Table 16. Lipid peroxidation products in plasma of two normal subjects after various periods of storage at -20°C.

Each value represents the mean of triplicate estimations ± S.D.

Storage Period (month)	CONJUGATED DIENE A_{235} ml^{-1} plasma		MALONDIALDEHYDE nmol ml^{-1} plasma		FLUORESCENT PIGMENT RFU ml^{-1} plasma	
	SAMPLE A	SAMPLE B	SAMPLE A	SAMPLE B	SAMPLE A	SAMPLE B
0	1.20±0.07	1.25±0.11	4.12±0.20	4.32±0.50	12.0±1.4	14.0±1.2
1	1.20±0.07	1.25±0.11	4.12±0.10	4.32±0.48	12.0±2.1	14.0±1.8
2	1.21±0.08	1.26±0.09	4.18±0.31	4.36±0.41	14.0±1.6	14.1±1.3
3	1.22±0.04	1.26±0.12	4.22±0.43	4.52±0.42	14.2±1.8	15.3±1.2
4	1.22±0.04	1.26±0.08	4.30±0.44	4.64±0.16	15.0±1.6	18.4±1.2
5	1.22±0.06	1.24±0.08	4.46±0.23	4.68±0.44	16.3±1.4	21.3±1.6
6	1.21±0.08	1.21±0.06	4.65±0.56	4.79±0.36	17.0±1.6	25.0±1.5

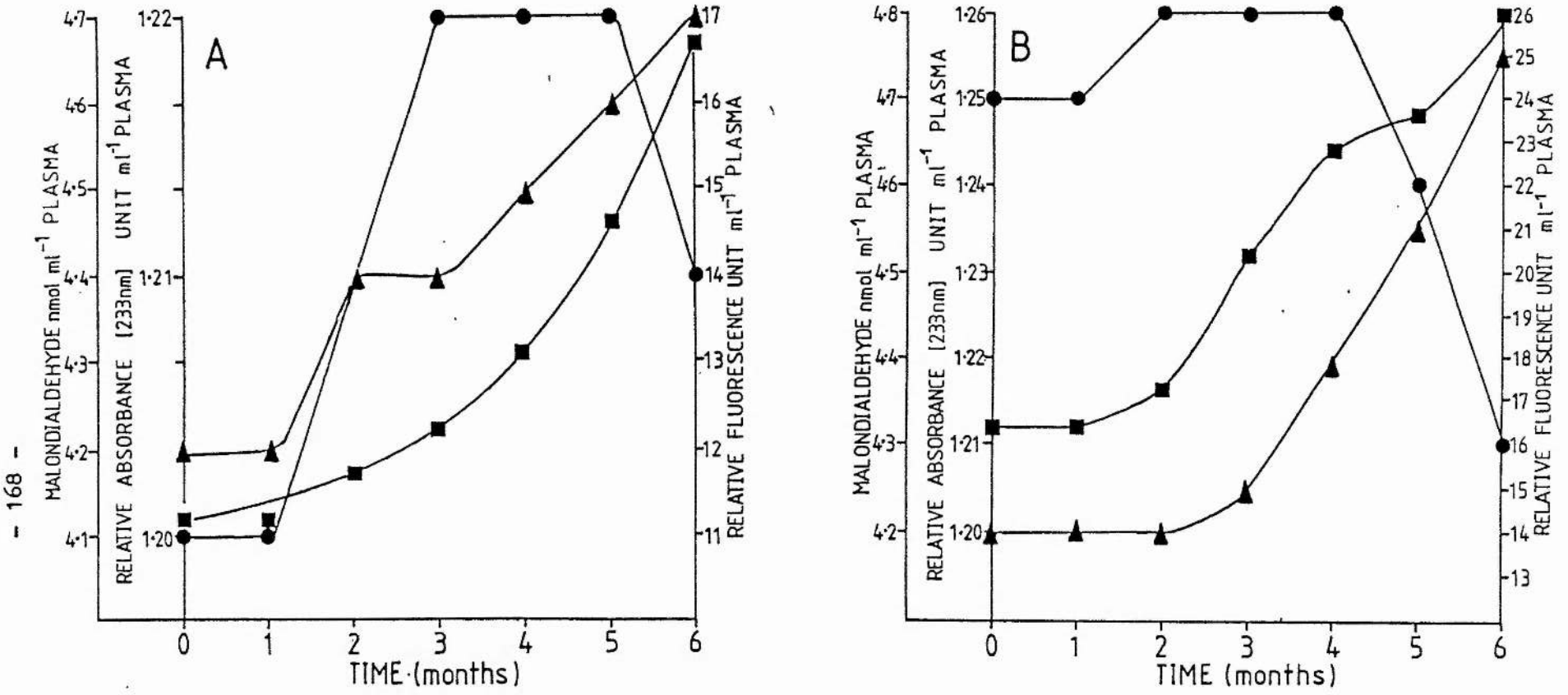


Figure 54. Variation in lipid peroxidation product concentration in normal plasma of subject A and B with storage time. Each point represents the mean of triplicate assays on an individual subject A or B.

●—● , conjugated diene.

■—■ , malondialdehyde.

▲—▲ , fluorescent pigment.

3.2.2 ASSAY OF PLASMA ANTIOXIDANTS

3.2.2.1 TOTAL PLASMA ANTIOXIDANT ACTIVITY (AOA)

Lipid peroxidation (autoxidation) occurs readily in a variety of tissue homogenates when they are incubated aerobically (Barber, 1961). The rate of formation of lipid peroxides, as measured by MDA production, is greatest in brain and hepatic tissue but significant quantities are also formed during incubation of other parentchymatous tissue such as testis, kidney and muscle (Barber,1961; McMurray and Dormandy, 1974). In this study a brain homogenate was chosen as the substrate material to investigate AOA in DMD and normal control plasma since it has the following advantages:-

(i) Fresh ox-brain can be readily obtained in large quatities from the local slaughter house.

(ii) Blood can easily be removed by removal of the meninges and washing in saline so that brain homogenate is ideally suited for preparation of a large single batch of homogenate for use as a substrate. This is a very important requirement for reproducible the measurement of the AOA of plasma.

(iii) Lastly, Donnan (1950) and Barber (1961) reported that lipid peroxidation in brain homogenates occurs rapidly during incubation at 37°C, the rate in this homogenate being greater compared to most other tissues. The high content of lipid and more importantly a relatively high content of the PUFA, arachidonic and docosahexaenoic acids, provide another advantage of using this tissue. It is these fatty acids which autoxidise most readily in tissue preparations (May *et*

al.,1965).

The effect of pooled plasma on the lipid autoxidation of standard brain homogenate is illustrated in Fig. 55 and Table 17. There was a significant difference between the AOA of DMD and normal control plasma ($P<0.001$). The AOA of DMD plasma was $76.3\pm2.0\%$ (mean$\pm$S.D, n=6) and that of normal control plasma was $62.9\pm4.9\%$ (mean+S.D, n=11). Also, with the limited number of patients studied were there was no overlap between DMD and control values. As shown in Table 18 there no significant correlation ($P>0.05$) was found between AOA and lipid peroxidation products in normal plasma In DMD plasma, a correlation coefficient of 0.78 was found between AOA and MDA concentration which indicates that they were highly significantly positively correlated ($P<0.05$).

Table 17. Plasma antioxidant activity from patients with DMD and normal control subjects.

Each value represents the mean of duplicate estimation ± S.D.

NORMAL CONTROLS

No.	Patients Initial	$\frac{MDA\ (T_1) - MDA\ (T_o)}{MDA\ (C_1) - MDA\ (C_o)}$ MEAN ± S.D.	ΔOA %B=(1-A)x100
1	ML (1)	0.30±0.03	70
2	ML (2)	0.44±0.02	56
3	JBM	0.31±0.03	69
4	DL	0.36±0.04	64
5	JDB	0.32±0.01	68
6	DK	0.40±0.02	60
7	AG	0.42±0.03	58
8	LC	0.37±0.02	63
9	EJ	0.34±0.02	66
10	DS	0.43±0.04	57
11	MISH	0.39±0.03	61
n = 11 (NORMAL CONTROL)			62.9±4.9

Table 17 (Continued)

DUCHENNE MUSCULAR DYSTROPHY

No.	Patients Initial	$\frac{MDA\ (T_1) - MDA\ (T_o)}{MDA\ (C_1) - MDA\ (C_o)}$ MEAN ± S.D.	AOA %B=(1-A)x100
1	NRJ	0.21±0.03	79
2	DT	0.26±0.04	74
3	DC	0.25±0.05	75
4	SO	0.22±0.02	78
5	JT	0.23±0.01	77
6	MF	0.25±0.03	75
(n = 6)	DUCHENNE		76.3±2.0

MDA = nmol/ml
C_o = control (zero time)
C_1 = control (1 hour)
T_o = test (zero time)
T_1 = test (1 hour)

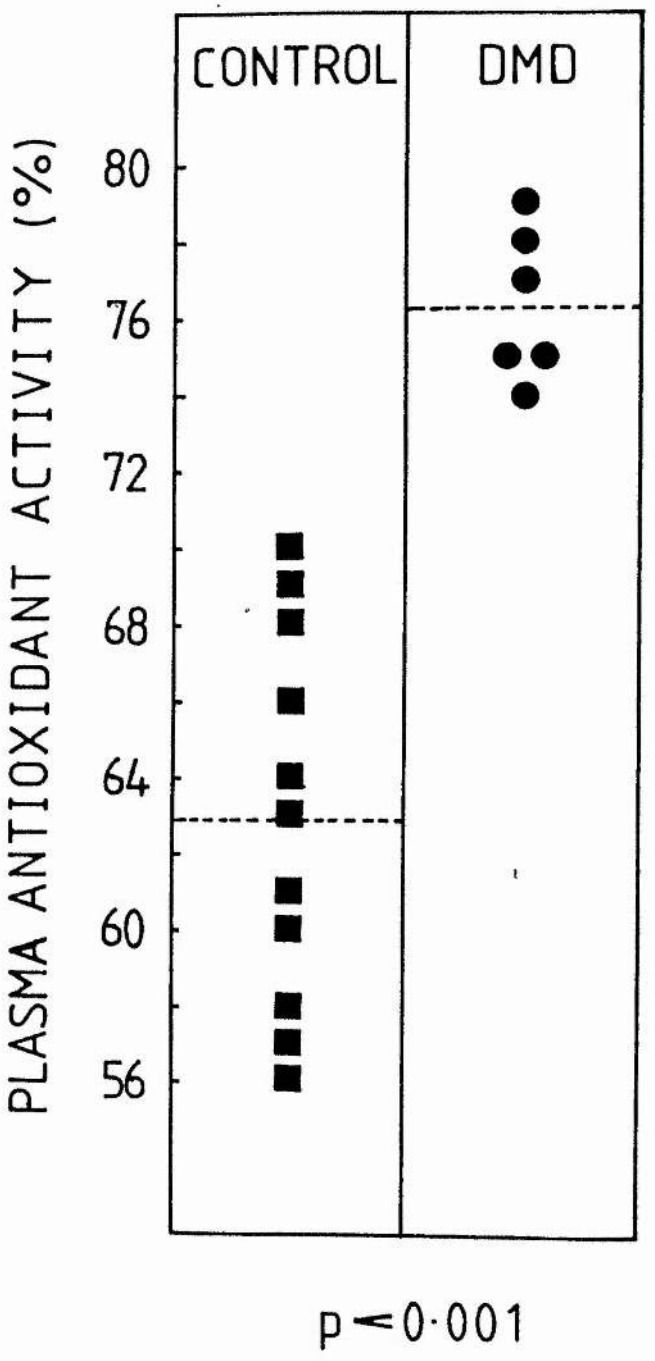


Figure 55. Distribution of antioxidant activity (%) in plasma of patients with DMD (●) and normal controls (■). Each point represents the mean of duplicate assays on an individual patient or control.

Table 18. Correlation coefficients between plasma antioxidant activity and lipid peroxidation products.

Indices Compared	Antioxidant Activity + Conjugated Diene		Antioxidant Activity + Malondialdehyde		Antioxidant Activity + Fluorescent Pigment	
Samples	Normal (n=11)	Duchenne (n=6)	Normal (n=11)	Duchenne (n=6)	Normal (n=11)	Duchenne (n=6)
Correlation Coefficients (r)	0.358	0.095	-0.113	0.781	-0.216	0.302
Probability (P)	NS	NS	NS	P<0.05*	NS	NS

NS = Not Significant

* = Significant correlation with antioxidant activity

3.2.2.2 PLASMA ALPHA-TOCOPHEROL (VITAMIN E) CONCENTRATION

A significant amount of evidence indicates that the primary, if not the sole; function of vitamin E in metabolism is that of an *in vivo* lipid antioxidant (as described earlier in Introduction). Probably the most direct evidence to substantiate this theory is that lipoperoxides have been found in the tissues of vitamin E-deficient animals (Dam and Granados, 1945). It is assumed that vitamin E acts as an *in vivo* lipid antioxidant, protecting PUFA in tissue lipids against peroxidation. The presence of low vitamin E concentration, especially in structural lipids, could result in widespread tissue damage.

In this study plasma vitamin E was estimated in DMD and age-matched normal control samples. As shown in Fig. 56 and Table 19 the mean plasma vitamin E was 4.14 ± 2.00 mg/litre plasma in DMD (n=8) and 8.12 ± 2.04 mg/litre plasma in age-matched normal controls (n=9). This difference was highly statistically significant ($P<0.002$).

Effect of storage ($-20^{\circ}C$) was also studied on plasma vitamin E level. The variation in plasma vitamin E concentration in DMD and normal control plasma over a period of up to 3 years is shown in Fig. 57 and Table 20. Because the study compared samples from different individuals stored over the 3 years period there is not as clear a picture as if the same samples had been monitored but a general decrease in alpha-tocopherol be seen in both DMD and control samples.

Table 19. Plasma alpha-tocopherol (vitamin E) concentration in DMD and normal control samples from adults and children.

NORMAL CONTROLS (ADULTS)

Patient Initials*	Vitamin E (mg/litre) (Mean ± S.D.)
MSL	10.09±0.47
MB	9.08±0.72
DK	9.52±0.24
AI	7.32±0.17
PR	10.20±0.07
AT	8.49±0.04
MISH	9.98±0.49
JQ	9.70±0.77
EJ	4.37±0.13
JDB	7.00±0.32
ML1	8.53±0.41
ML2	12.55±0.05
BCN	10.80±0.30
AL	8.50±0.30
JH	11.45±0.05
JM	5.80±0.50
PN	14.65±0.40
n = 17	9.30±2.45

* age between 18 and 35

Table 19. (Continued)

NORMAL CONTROLS (CHILDREN)

Patient Initials*	Vitamin E (mg/litre) (Mean ± S.D.)
PA	9.48±1.21
A	8.56±1.17
LH	7.66±1.76
MG	10.21±1.30
HT	6.20±0.81
FF	10.04±1.16
GR	10.04±1.40
AE	4.67±0.46
EP	6.20±1.24
n = 9	8.12±2.04

* age between 4 and 16

Table 19. (Continued)

DUCHENNE MUSCULAR DYSTROPHY

Patient Initials*	Vitamin E (mg/litre) (Mean ± S.D.)
DC	2.99±0.11
SO	2.81±0.76
JT	4.05±0.53
DT	5.20±0.03
NRJ	3.12±0.23
MF	2.64±0.43
JM	3.67±0.22
DR	8.67±1.22
n = 8	4.14±2.00

* age between 3 and 16

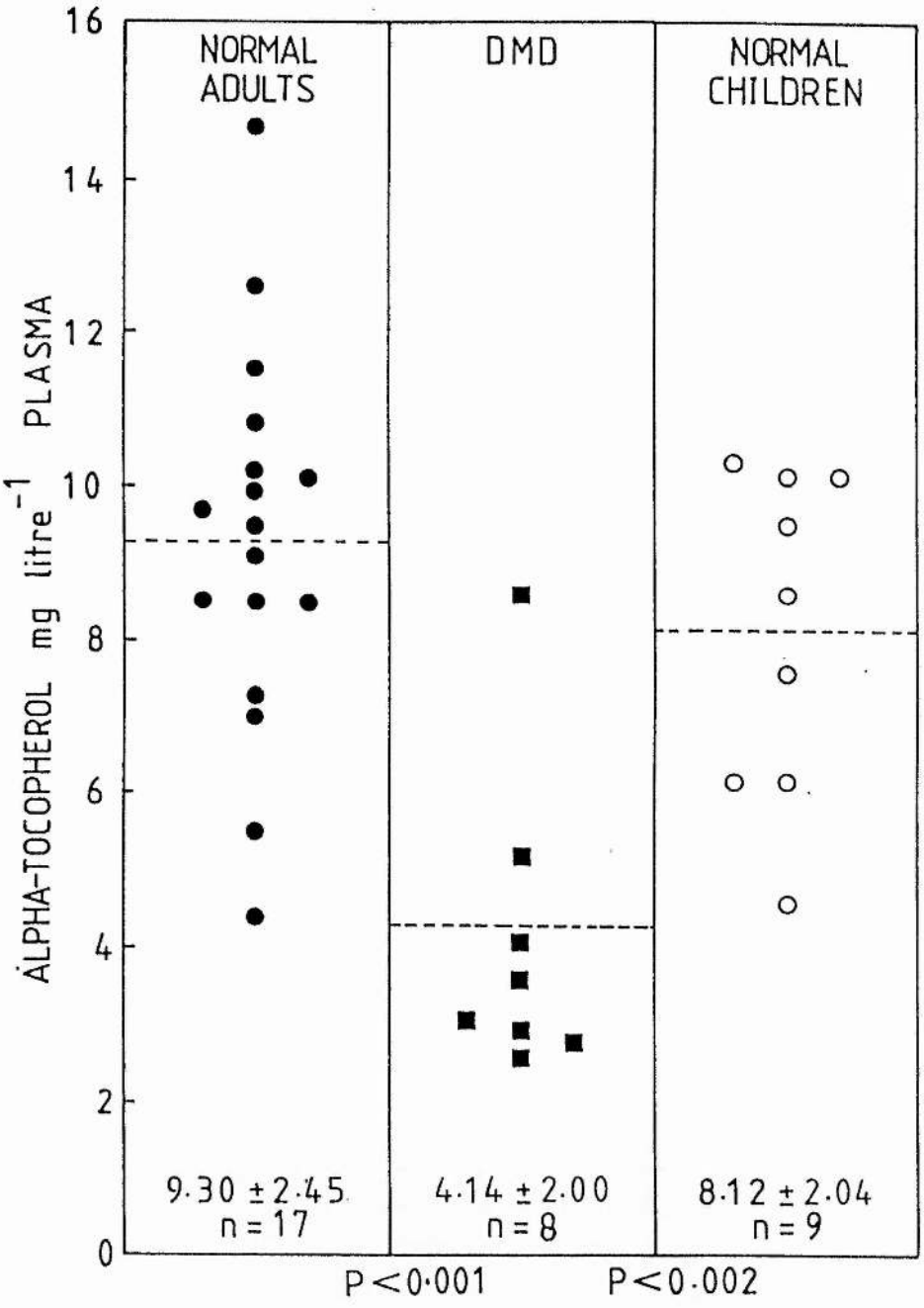


Figure 56. Distribution of alpha-tocopherol concentration in plasma of patients with DMD (■), normal adults (●) and normal children (○).

Table 20. Alpha-tocopherol concentration in plasma from patients with DMD and normal control subjects after storage at -20°C for different periods. Number of samples for different storage period are as follow: 0 (N^{*}=11, D^{**}=9), 0.5 (N=8, D=4), 1.0 (N=7, D=2), 2.0 (N=4, D=3) and 3.0 (N=8, D=3). Each value represents the mean for each group of subjects and assay were carried out in duplicate estimations ± S.D.

ALPHA-TOCOPHEROL mg $litre^{-1}$ PLASMA		
Storage time (years)	Normal Control (MEAN±S.D.)	Duchenne (MEAN±S.D.)
0	10.63±2.84	4.17±0.50
0.5	8.75±1.77	3.47±0.98
1·0	8.66±2.51	4.17±0.65
2·0	9.44±1.51	3.25±0.32
3·0	3.71±1.53	2.28±1.22

N^{*}= Normal control, D^{**}= Duchenne muscular dystrophy.

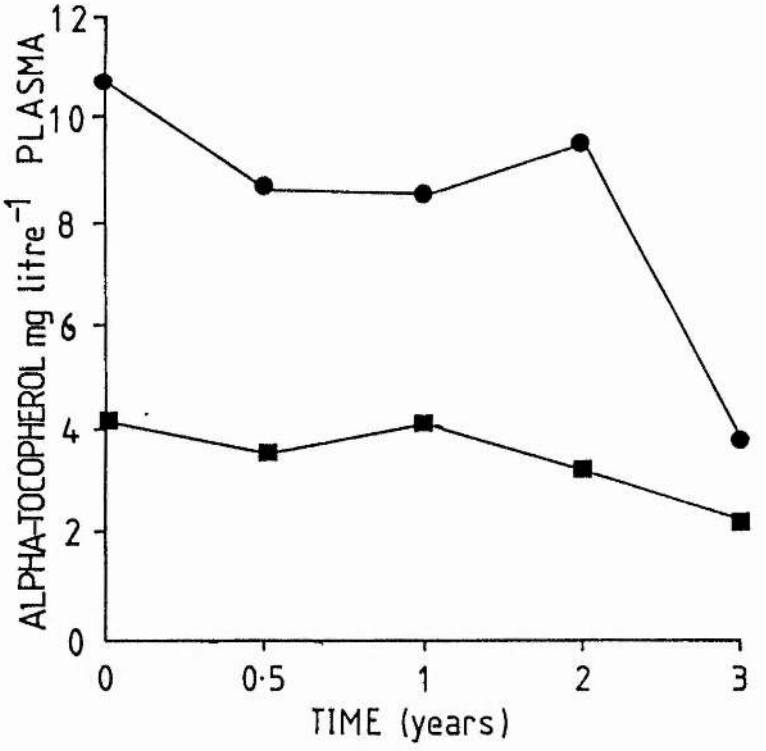


Figure 57. Alpha-tocopherol concentration in plasma at various storage times (-20°C), expressed as mg/litre plasma. Each point represents the mean for each group of subjects and assay were carried out in duplicate estimations. ●—●, control; ■—■, DMD.

This decrease seems to be fairly general between/and 3 years for the DMD samples (50% decrease) whereas for the controls the major loss appears to occur between 2 and 3 years (total 63% decrease). Since it is recognized that neonates often have lower vitamin E levels than adults, a group of healthy adults (age range 24-35 years) whose data is shown in Fig. 56 were compared with a group of 9 children (age range 9-16 years). As shown in Table 21, there was no significant different between between vitamin E level in adult and childrens plasma samples obtained by HPLC analysis ($P>0.05$).

In addition, Table 22 shows correlation coefficients between vitamin E and several other variables as listed in the table. No significant correlation between vitamin E and any variable in DMD and normal control plasma samples, was found.

Table 21. Comparison of alpha-tocopherol (vitamin E) concentration in normal children and adults with DMD patients.

TYPE OF SAMPLES	ALPHA-TOCOPHEROL mg litre^{-1} plasma
Normal Adults (n=17)	9.30±2.45
Normal Children (n=9)	8.12±1.92
Duchenne (n=8)	4.17±0.50

Table 22. Correlation coefficients between vitamin E and other variables.

Plasma Samples	Indices Compared	Correlation Coefficients (r)	Probability (P)
NORMAL CONTROL (n = 11)	E + CpRc	0.147	NS
	E + CpRa	0.241	NS
	E + TfRc	0.099	NS
	E + TfRa	-0.154	NS
	E + CD	-0.177	NS
	E + MDA	-0.436	NS
	E + FP	0.446	NS
	E + AOA	-0.329	NS
DUCHENNE (n = 6)	E + CpRc	0.241	NS
	E + CpRa	0.147	NS
	E + TfRc	0.091	NS
	E + TfRa	-0.154	NS
	E + CD	-0.356	NS
	E + MDA	-0.407	NS
	E + FP	-0.218	NS
	E + AOA	0.336	NS

E = Vitamin E (alpha-tocopherol), CpRc = Caeruloplasmin (Rocket), CpRa = Caeruloplasmin (Radial), TfRc = Transferrin (Rocket), RfTa = Transferrin (Radial), CD = Conjugated diene, MDA = Malondialdehyde, FP = Fluorescent pigment, AOA = Antioxidant activity, NS = Not significant.

3.2.2.3 PLASMA CAERULOPLASMIN CONCENTRATION

Caeruloplasmin concentrations in plasma of patients with DMD and age-matched normal controls were measured by immunological methods (radial immunodiffusion and rocket immunoelectrophoresis). Results are shown in Table 23 and Figs. 58-59. DMD plasma showed a highly significant increase in the content of caeruloplasmin as measured by radial immunodiffusion (100.75±11.97 I.U/ml plasma, $P<0.002$) (Fig. 58), and by rocket immunoelectrophoresis (98.16±11.24 I.U/ml plasma, $P<0.001$) (Fig 59). Normal control (age-matched) plasma contained 79.12±11.90 I.U/ml plasma as determined by radial immunodiffusion and 75.82±8.33 I.U/ml plasma by rocket immunoelctrophoresis.

Possible correlation of DMD plasma caeruloplasmin concentration against several other variables (as listed) was tested and the result is shown in Table 24a and 24b. Interestingly, in DMD, a significant correlation between caeruloplasmin concentration (measured by both radial and rocket immunoelectrophoresis) and AOA and also with two of the lipid peroxidation products measured (MDA and FP) was found. The significance tended to be greater for DMD plasma. A correlation coefficient of 0.868 was found between caeruloplasmin content measured by rocket immunoelectrophoresis and MDA concentration which indicates that they were very significantly positively correlated ($P<0.001$). This may be an important finding whose significance is dealt with in the discussion.

Table 23. Plasma caeruloplasmin content in normal adults, children and DMD patients.

NORMAL CONTROLS (ADULTS)

Patient Initials*	CAERULOPLASMIN (I.U./~~LITRE~~) ml (MEAN ± S.D.)	
	Radial Immunodiffusion	Rocket Immunoelectrophoresis
MSL	39.41±7.31	80.00±8.43
MB	65.94±8.82	73.60±7.31
DK	84.75±9.90	73.60±11.11
AI	94.70±10.80	67.20±8.71
PR	70.40±9.21	71.68±9.36
AT	61.40±8.61	83.20±8.42
MISH	71.40±9.16	85.61±6.72
JBM	77.47±9.32	70.54±12.11
PN	60.80±18.62	84.33±11.14
DL	77.22±9.46	76.63±8.47
AG	81.60±9.52	89.78±9.54
LC	81.82±9.74	76.63±10.14
DS	99.80±10.60	78.72±12.46
JQ	70.40±9.21	69.76±8.41
EJ	91.43±9.58	79.20±7.42
ML1	77.42±9.36	76.63±9.11
ML2	79.82±19.48	75.67±11.10
JDB	86.45±19.48	89.14±9.46
BCN	87.10±10.10	86.40±9.46
AL	62.70±8.70	89.28±12.10
JH	65.60±8.81	88.96±9.82
JM	70.40±9.21	75.67±6.48
n = 22	75.37±13.49	79.19±6.96

* age between 18 and 35

Table 23. (Continued)

NORMAL CONTROLS (CHILDREN)

Patient Initials*	CAERULOPLASMIN (I.U./~~LITRE~~) ml (MEAN ± S.D.)	
	Radial Immunodiffusion	Rocket Immunoelectrophoresis
PA	62.10±8.54	76.63±9.74
A	79.10±9.54	69.76±10.13
LH	85.80±9.92	73.60±9.41
MG	77.40±9.51	71.68±10.14
HT	82.60±9.82	67.20±12.16
FF	99.80±10.60	71.68±9.32
GR	62.70±8.70	89.14±9.48
AE	70.40±9.21	67.12±10.08
EP	91.43±9.58	89.28±8.88
GP	79.82±9.48	82.14±7.41
n = 10	79.12±11.90	75.82±8.33

* age between 4 and 16

Table 23. (Continued)

DUCHENNE MUSCULAR DYSTROPHY

Patient Initials*	CAERULOPLASMIN (I.U./LITRE) (MEAN ± S.D.)	
	Radial Immunodiffusion	Rocket Immunoelectrophoresis
DC	91.52±8.80	89.16±9.32
SO	93.44±8.84	98.12±8.32
JT	97.28±9.24	98.12±10.04
DT	93.44±8.82	84.12±9.41
NRJ	86.40±8.42	82.26±9.36
MF	118.50±8.96	110.62±8.41
JM	97.28±8.85	102.02±9.23
DR	118.50±11.40	114.60±10.14
PM	110.40±9.36	104.41±8.13
n = 9	100.75±11.97	98.16±11.24

* age between 3 and 16

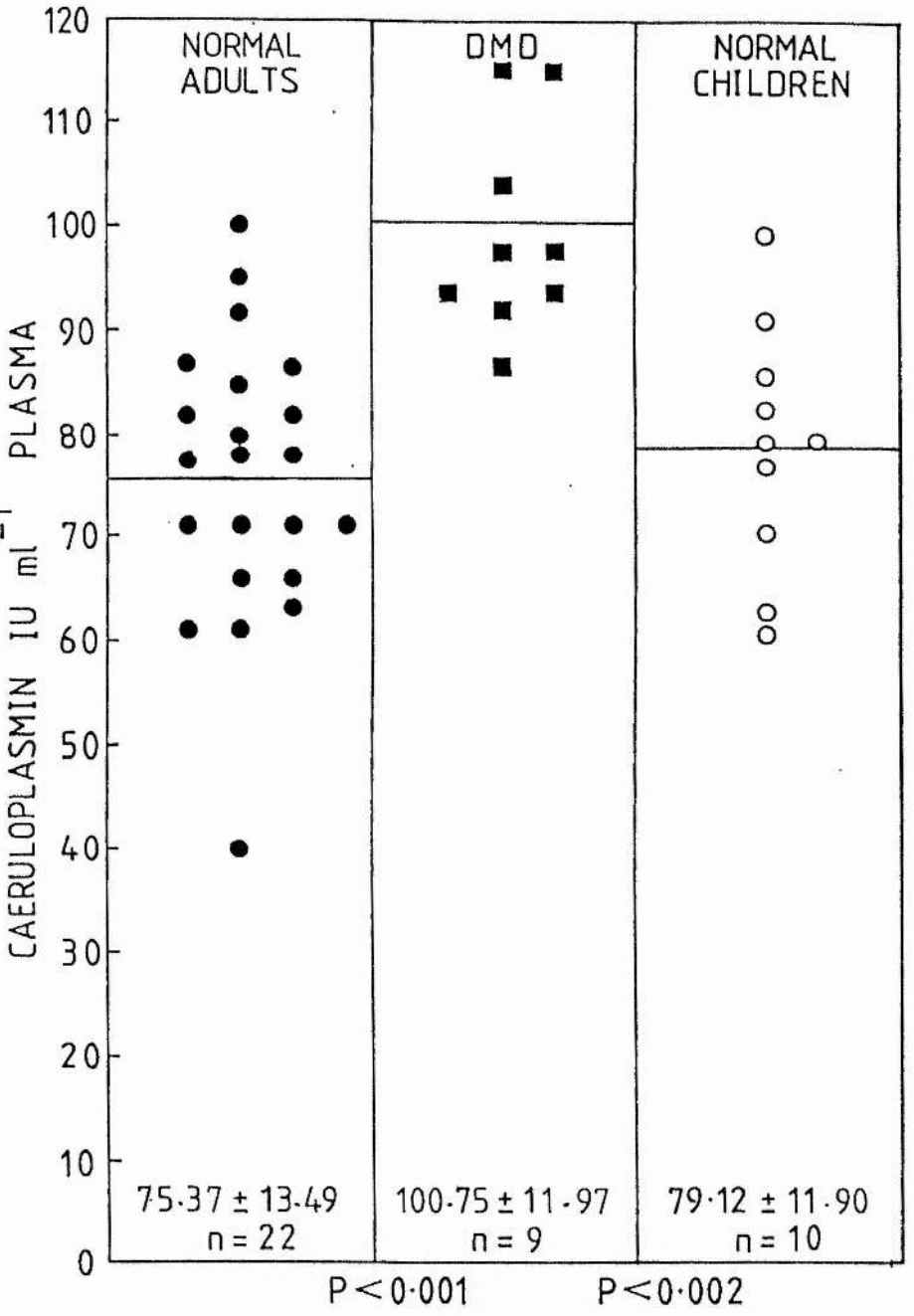


Figure 58. Distribution of caeruloplasmin in plasma of patients with DMD (■) and controls (●,○) measured by radial immunodiffussion. Each point represents the mean of triplicate assays on an individual patient or control.

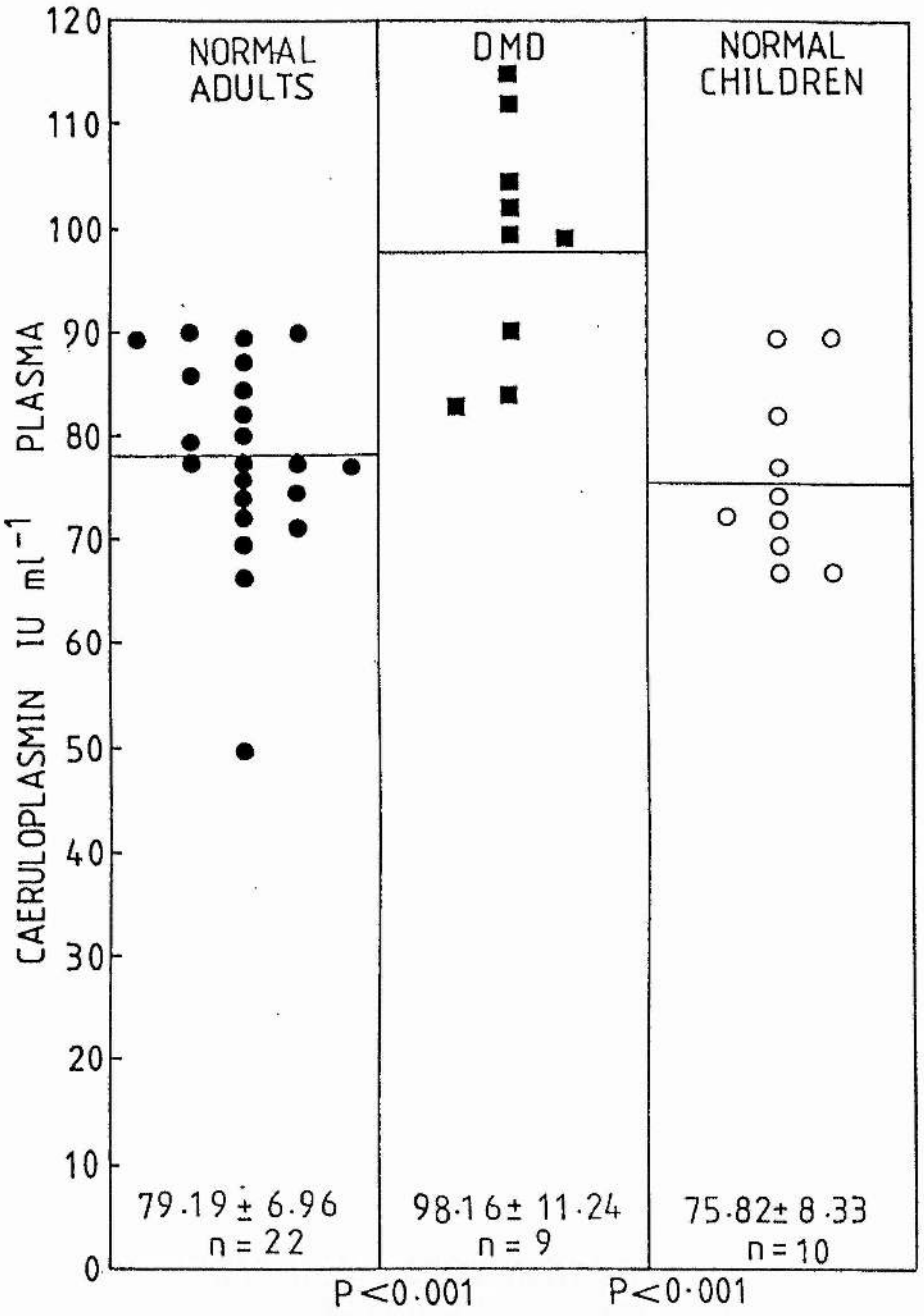


Figure 59. Distribution of caeruloplasmin in plasma of patients with DMD (■) and controls (●,○) measured by rocket immunoelectrophoresis. Each point represents the mean of triplicate assays on an individual patient or control.

Table 24a. Correlation coefficents between caeruloplasmin (radial) and other variables.

Plasma Samples	Indices Compared	Correlation Coefficients (r)	Probability (P)
NORMAL CONTROL (n = 11)	CpRa + TfRc	-0.034	NS
	CpRa + TfRa	-0.034	NS
	CpRa + FP	0.359	NS
	CpRa + MDA	-0.421	NS
	CpRa + CD	-0.383	NS
	CpRa + AOA	-0.232	NS
	CpRa + CpRc	-0.366	NS
DUCHENNE (n = 6)	CpRa + TfRc	-0.348	NS
	CpRa + TfRa	-0.359	NS
	CpRa + FP	0.546	NS
	CpRa + MDA	-0.858	P<0.05*
	CpRa + CD	-0.338	NS
	CpRa + AOA	0.738	P<0.05*
	CpRa + CpRc	0.885	P<0.01*

CpRa = Caeruloplasmin (Radial), CpRc = Caeruloplasmin (Rocket), TfRa = Transferrin (Radial), TfRc = Transferrin (Rocket), CD = Conjugated diene, MDA = Malondialdehyde, FP = Fluorescent pigment, AOA = Antioxidant activity, NS = Not significant, * = significant correlation with caeruloplasmin (Radial) concentration.

Table 24b. Correlation coefficients between caeruloplasmin (rocket) and other variables.

Plasma Samples	Indices Compared	Correlation Coefficients (r)	Probability (P)
NORMAL CONTROL (n = 11)	CpRc + TfRc	0.281	NS
	CpRc + TfRa	0.547	NS
	CpRc + FP	-0.541	NS
	CpRc + MDA	0.421	NS
	CpRc + CD	0.042	NS
	CpRc + AOA	-0.125	NS
	CpRc + CpRa	-0.366	NS
DUCHENNE (n = 6)	CpRc + TfRc	0.322	NS
	CpRc + TfRa	-0.537	NS
	CpRc + FP	0.726	P<0.05*
	CpRc + MDA	0.868	P<0.01*
	CpRc + CD	-0.397	NS
	CpRc + AOA	0.711	P<0.05*
	CpRc + CpRa	0.885	P<0.01*

CpRa = Caeruloplasmin (Radial), CpRc = Caeruloplasmin (Rocket), TfRa = Transferrin (Radial), TfRc = Transferrin (Rocket), CD = Conjugated diene, MDA = Malondialdehyde, FP = Fluorescent pigment, AOA = Antioxidant activity, NS = Not significant, * = significant correlation with caeruloplasmin (rocket) concentration.

The effect of age on caeruloplasmin content was also investigated, and the result is shown in Table 25. Both method gave no significant different between the caeruloplasmin content in plasma from adults and children (radial = P>0.05 and rocket=P>0.05).

The effects of storage (-20^{o}C) on caeruloplasmin content are shown in Figs. 60-61 and Table 26. Clearly from these graphs, time of storage did not influence the level of caeruloplasmin in either DMD or normal control plasma, measured by both radial immunodiffusion (Fig. 60) and rocket immunoelectrophoresis (Fig. 61). The small fluctuations in content of cearuloplasmin with time are probably due to the heterogeneity and small number of the samples used.

Table 25. Comparison of plasma caeruloplasmin concentration in normal children and adults with DMD patients.

	CAERULOPLASMIN	
Type of Samples	RADIAL IMMUNODIFFUSION (I.U./ml Plasma) (MEAN ± S.D.)	ROCKET IMMUNOELECTROPHORESIS (I.U./ml Plasma) (MEAN ± S.D.)
Normal Adults (n = 22)	75.37 ± 13.49	79.19 ± 6.96
Normal Children (n = 10)	79.12 ± 11.90	75.82 ± 8.33
Duchenne (n = 9)	100.75 ± 11.97	98.16 ± 11.24

Table 26. Caeruloplasmin concentration in plasma from patients with DMD and normal control subjects after various times of storage at -20°C. Number of samples for different storage period are as follow: 0 (N^{*}=22, D^{**}=9), 0.5 (N=5, D=4), 1.0 (N=6, D=2), 2.0 (N=4, D=3) and 3.0 (N=5, D=3). Each value represents the mean for each group of subjects and assay were carried out in duplicate estimations ± S.D.

Storage Time (Years)	CAERULOPLASMIN			
	RADIAL IMMUNODIFFUSION (I.U./ml Plasma)		ROCKET IMMUNOELECTROPHORESIS (I.U./ml Plasma)	
	NORMAL (MEAN ±S.D.)	DUCHENNE (MEAN ± S.D.)	NORMAL (MEAN ±S.D.)	DUCHENNE (MEAN ±S.D.)
0	75.37±13.49	96.76±10.25	79.19±6.96	93.73±9.74
0.5	79.12±11.29	98.76±12.64	71.62±8.46	92.12±12.46
1	62.83±12.74	95.66±20.32	64.15±9.03	88.38±17.27
2	68.10±7.36	98.59±18.68	67.84±8.19	92.90±15.05
3	68.18±8.39	91.16±17.80	81.02±6.51	93.78±11.05

N^{*} = Normal control; D^{**} = Duchenne muscular dystrophy.

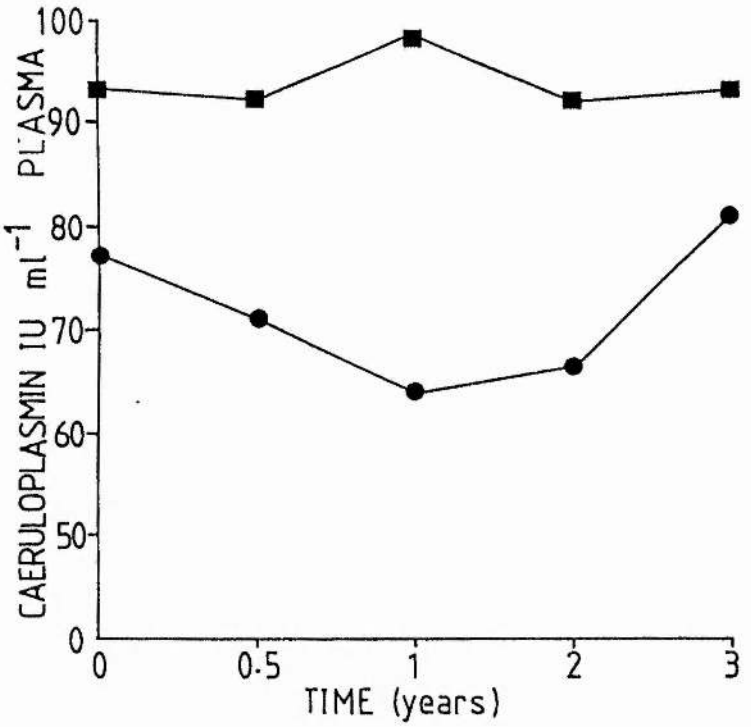


Figure 60. Caeruloplasmin concentration in plasma after various times of storage at -20°C measured by radial immunodiffusion. Each point represents the mean for each group of subjects and assay were carried out in duplicate estimations. ●—●, control; ■—■, DMD.

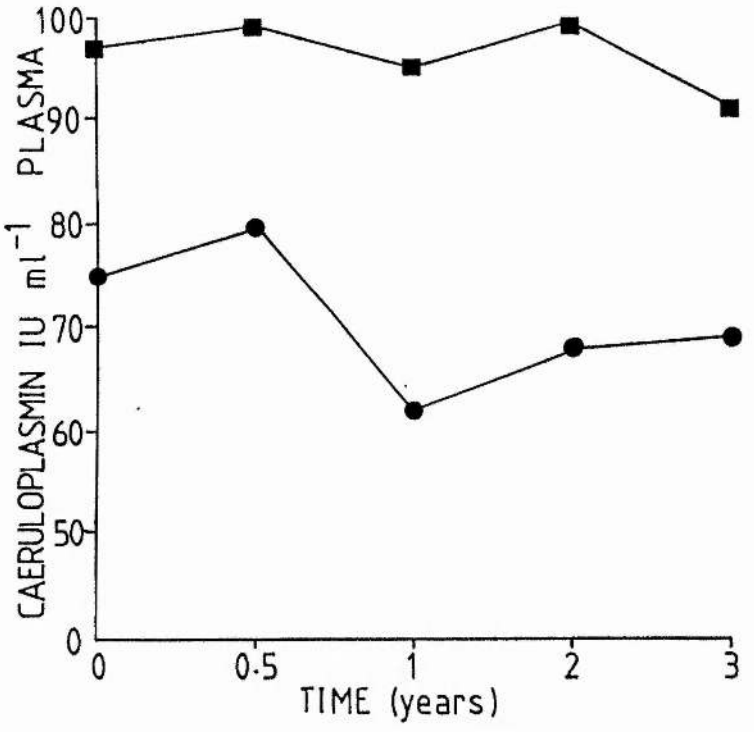


Figure 61. Caeruloplasmin concentration in plasma after various times of storage at -20°C measured by rocket immunoelectrophoresis. Each point represents the mean for each group of subjects and assay were carried out in duplicate estimations. ●——●, control; ■——■, DMD.

3.2.2.4 PLASMA TRANSFERRIN CONCENTRATION

In contrast to caeruloplasmin the concentration of transferrin in DMD plasma was not significantly different from age-matched normal controls (Table 27) as measured by radial immunodiffusion (176.89±58.80 I.U/ml plasma, $P>0.05$) (Fig. 62) and rocket immunoelectrophoresis (171.33±53.36 I.U/ml plasma, $P>0.05$) (Fig. 63). Normal control (age-matched) contained 156.00±75.64 I.U/ml plasma by radial immunodiffusion and 158.00±72.79 I.U/ml plasma by rocket immunoelectrophoresis.

Again, possible correlation of plasma transferrin against several other variables (as listed) was tested. The results are shown in Table 28a and 28b. As expected the correlation between transferrin measured by radial immunodiffusion and rocket immunoelectrophoresis was significant in both DMD and normal control samples. This differed from the finding for caeruloplasmin where there was no correlation between radial and rocket immunoelectrophoresis for control. However, there was no significant correlation between transferrin concentration (by either radial or rocket) and the listed variables for both DMD and control samples.

As for caeruloplasmin, the effect of age on transferrin concentration was studied as shown in Table 29. Neither radial immunodiffusion nor rocket immunoelectrophoresis showed a significant difference ($P>0.05$) between adults and children.

Table 27. Plasma transferrin content in DMD and normal control samples from adults and children.

NORMAL CONTROLS (ADULTS)

Patient Initials*	TRANSFERRIN (I.U./LITRE) (MEAN ± S.D.)	
	Radial Immunodiffusion	Rocket Immunoelectrophoresis
MSL	142.2±9.6	134.8±11.4
MB	123.8±8.4	109.2±9.6
DK	209.4±10.4	182.4±15.6
AI	182.6±10.1	192.6±16.4
PR	272.5±11.2	208.2±17.8
AT	121.5±8.1	122.0±10.4
MISH	99.9±7.4	100.0±8.5
JBM	86.1±7.0	80.4±6.9
PN	154.2±10.2	106.8±12.4
DL	147.8±11.2	140.4±16.2
AG	274.6±10.4	248.3±8.2
LC	204.2±8.6	209.6±11.2
DS	96.2±9.2	100.4±9.4
JQ	184.8±8.0	149.6±12.8
EJ	272.6±9.1	185.6±16.0
ML1	208.4±10.1	193.4±12.3
ML2	133.6±11.1	148.2±8.4
JDB	150.4±9.4	152.6±11.4
BCN	121.4±9.0	119.8±13.1
AL	149.4±9.1	122.1±10.4
JA	182.6±10.1	145.9±12.3
JM	242.8±9.4	209.7±18.0
n = 22	171.2 ± 57.4	152.8 ± 44.9

* age between 18 and 35

Table 27. (Continued)

NORMAL CONTROLS (CHILDREN)

Patient Initials*	TRANSFERRIN (I.U./LITRE) (MEAN ± S.D.)	
	Radial Immunodiffusion	Rocket Immunoelectrophoresis
PA	87.6±7.0	96.4±8.2
A	121.8±8.4	110.7±9.6
LH	87.4±7.7	92.3±7.7
MG	121.3±10.4	146.8±12.3
HT	278.6±20.3	285.3±24.4
FF	283.7±26.4	276.4±23.2
GR	213.5±16.2	183.6±15.8
AE	147.2±16.4	171.7±14.8
EP	98.5±8.4	85.6±7.2
GR	120.4±10.6	131.2±11.3
n = 10	156.0 ± 75.6	158.0 ± 72.7

* age between 4 and 16

Table 27. (Continued)

DUCHENNE MUSCULAR DYSTROPHY

Patient Initials*	TRANSFERRIN (I.U./LITRE) (MEAN ± S.D.)	
	Radial Immunodiffusion	Rocket Immunoelectrophoresis
DC	283.2±18.6	240.3±20.4
SD	157.4±9.8	150.6±12.3
JT	223.4±18.4	245.7±20.6
DT	147.1±14.1	142.4±12.0
NRJ	220.6±16.4	209.7±18.0
MF	96.0±9.4	85.4±7.2
JM	180.8±20.4	185.3±16.1
DR	109.9±13.4	124.6±10.6
PM	173.6±24.1	158.0±27.6
n = 9	176.8 ± 58.8	171.3 ± 53.3

* age between 3 and 16

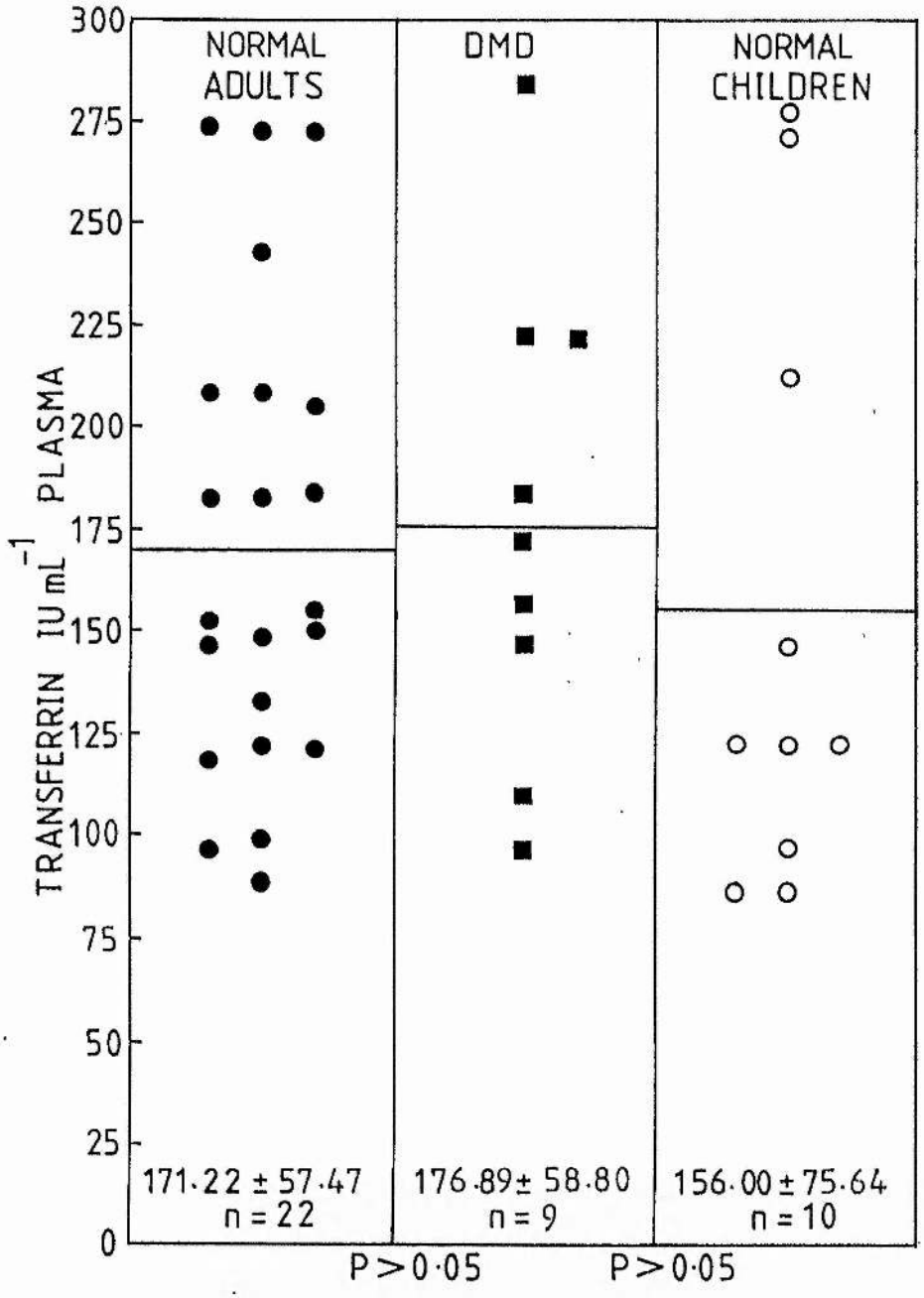


Figure 62. Distribution of transferrin in plasma of patients with DMD (■) and controls (●,○) measured by radial immunodiffusion. Each point represents the mean of triplicate assays on an individual patient or control.

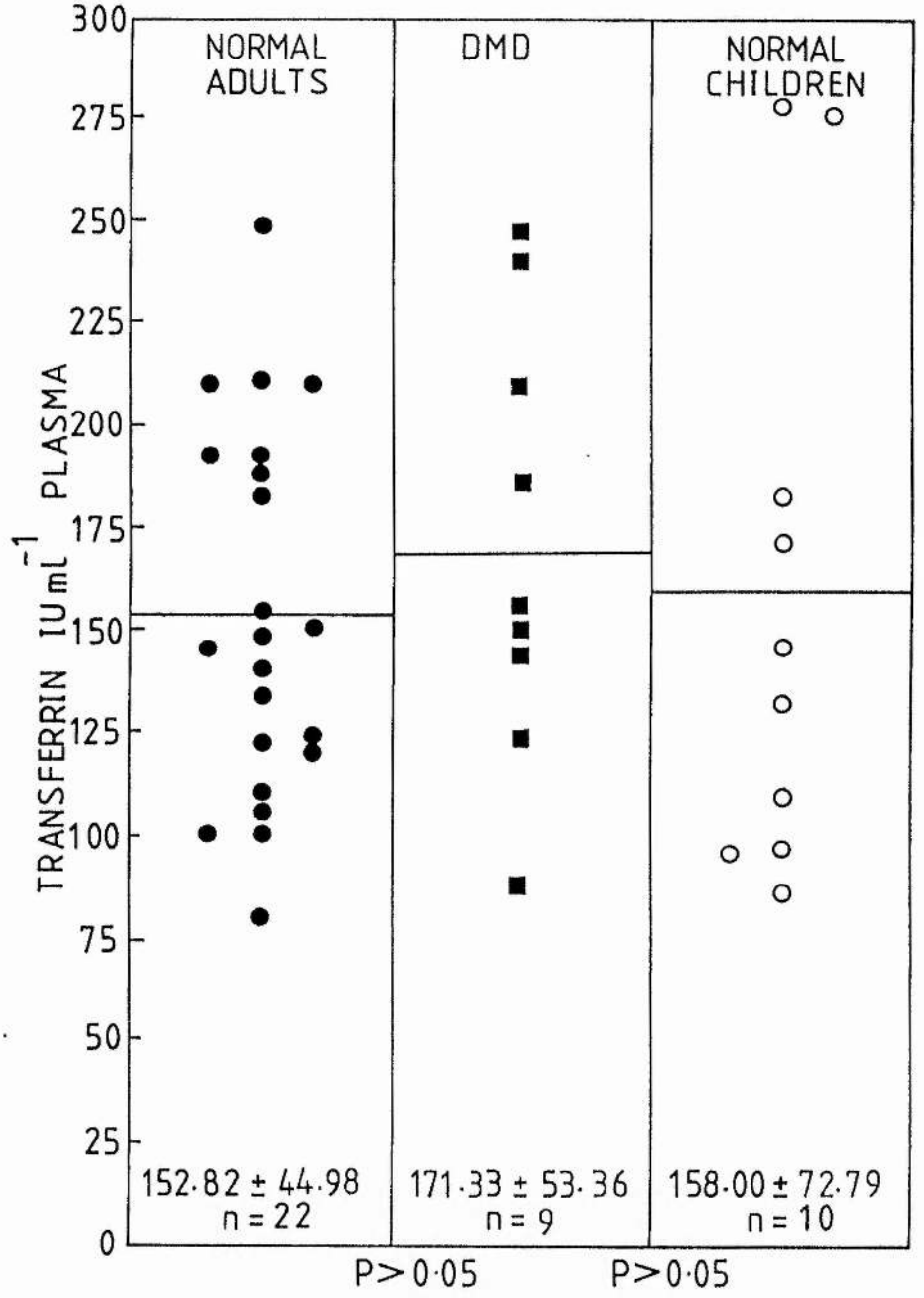


Figure 63. Distribution of transferrin in plasma of patients with DMD (■) and controls (●,○) measured by rocket immunoelectrophoresis. Each point represents the mean of triplicate assays on an individual patient or control.

Table 28a. Correlation coefficients between transferrin (radial) and other variables.

Plasma Samples	Indices Compared	Correlation Coefficients (r)	Probability (P)
NORMAL CONTROL (n = 11)	TfRa + CpRc	-0.547	NS
	TfRa + CpRa	-0.034	NS
	TfRa + FP	-0.139	NS
	TfRa + MDA	-0.203	NS
	TfRa + CD	-0.221	NS
	TfRa + AOA	0.195	NS
	TfRa + TfRc	0.595	P<0.05*
DUCHENNE (n = 6)	TfRa + CpRc	-0.537	NS
	TfRa + CpRa	-0.359	NS
	TfRa + FP	-0.654	NS
	TfRa + MDA	-0.619	NS
	TfRa + CD	0.161	NS
	TfRa + AOA	0.539	NS
	TfRa + TfRc	0.951	P<0.001*

TfRa = Transferrin (Radial), TfRc = Transferrin (Rocket), CpRa = Caeruloplasmin (Radial), CpRc = Caeruloplasmin (Rocket), CD = Conjugated diene, MDA = Malondialdehyde, FP = Fluorescent pigment, AOA = Antioxidant activity, NS = Not significant, * = Significant correlation with transferrin (radial) concentration.

Table 28b. Correlation coefficient between transferrin (rocket) and other variables.

Plasma Samples	Indices Compared	Correlation Coefficients (r)	Probability (P)
NORMAL CONTROL (n = 11)	TfRc = CpRc	0.281	NS
	TfRc + CpRa	-0.034	NS
	TfRc + FP	-0.251	NS
	TfRc + MDA	-0.096	NS
	TfRc + CD	-0.194	NS
	TfRc + AOA	-0.669	NS
	TfRc + TfRa	0.595	P<0.05*
DUCHENNE (n = 6)	TfRc + CpRc	0.322	NS
	TfRc + CpRa	-0.348	NS
	TfRc + FP	-0.714	NS
	TfRc + MDA	-0.591	NS
	TfRc + CD	0.121	NS
	TfRc + AOA	0.363	NS
	TfRc + TfRa	0.951	P<0.001*

TfRa = Transferrin (Radial), TfRc = Transferrin (Rocket), CpRa = Caeruloplasmin (Radial), CpRc = Caeruloplasmin (Rocket), CD = Conjugated diene, MDA = Malondialdehyde, FP = Fluorescent pigment, AOA = Antioxidant activity, NS = Not signficant, * = Significant correlation with transferrin (rocket) concentration.

Table 29. Comparison of plasma transferrin concentration in normal children and adults with DMD patients.

	TRANSFERRIN	
Type of Samples	RADIAL IMMUNODIFFUSION (I.U./ml Plasma) (MEAN ± S.D.)	ROCKET IMMUNOELECTROPHORESIS (I.U./ml Plasma) (MEAN ± S.D.)
Normal Adults (n = 22)	171.2 ± 57.4	152.8 ± 44.9
Normal Children (n = 10)	156.0 ± 75.6	158.0 ± 72.7
Duchenne (n = 9)	176.8 ± 58.8	171.3 ± 53.3

Table 30. Transferrin concentration in plasma from patients with DMD and normal control subjects after various times of storage at -20°C. Number of samples for different storage period are as follow: 0 (N^{*}=22, D^{**}=9), 0.5 (N=5, D=4), 1.0 (N=8, D=2), 2.0 (N=4, D=3) and 3.0 (N=9, D=3). Each value represents the mean for each group of subjects and assay were carried out in duplicate estimations ± S.D.

	TRANSFERRIN			
	RADIAL IMMUNODIFFUSION (I.U./ml Plasma)		ROCKET IMMUNOELECTROPHORESIS (I.U./ml Plasma)	
Storage Time (Years)	NORMAL (MEAN ± S.D.)	DUCHENNE (MEAN ± S.D.)	NORMAL (MEAN ± S.D.)	DUCHENNE (MEAN ± S.D.)
0	171.2 ± 57.4	187.8 ± 61.6	152.8 ± 44.9	178.8 ± 57.5
0.5	173.6 ± 41.6	178.8 ± 57.4	162.7 ± 58.1	176.4 ± 43.1
1	165.0 ± 37.9	167.3 ± 54.6	157.8 ± 34.9	180.3 ± 53.7
2	155.0 ± 33.2	178.0 ± 79.2	161.5 ± 31.4	174.0 ± 71.8
3	177.8 ± 40.4	180.1 ± 60.8	174.5 ± 43.6	177.7 ± 47.3

N^{*} = Normal control; D^{**} = Duchenne muscular dystrophy.

Lastly, the effect of storage (-20°C) was also investigated. Results is shown in Table 30 and Figs. 64-65. Clearly from these graphs, the concentration of transferrin was not altered by storage as observed for caeruloplasmin. Both radial immunodiffusion (Fig. 64) and rocket immunoelectrophoresis (Fig. 65) were used and the reasons for the considerable fluctuations seen with storage time are probably the same as those suggested earlier for caeruloplasmin.

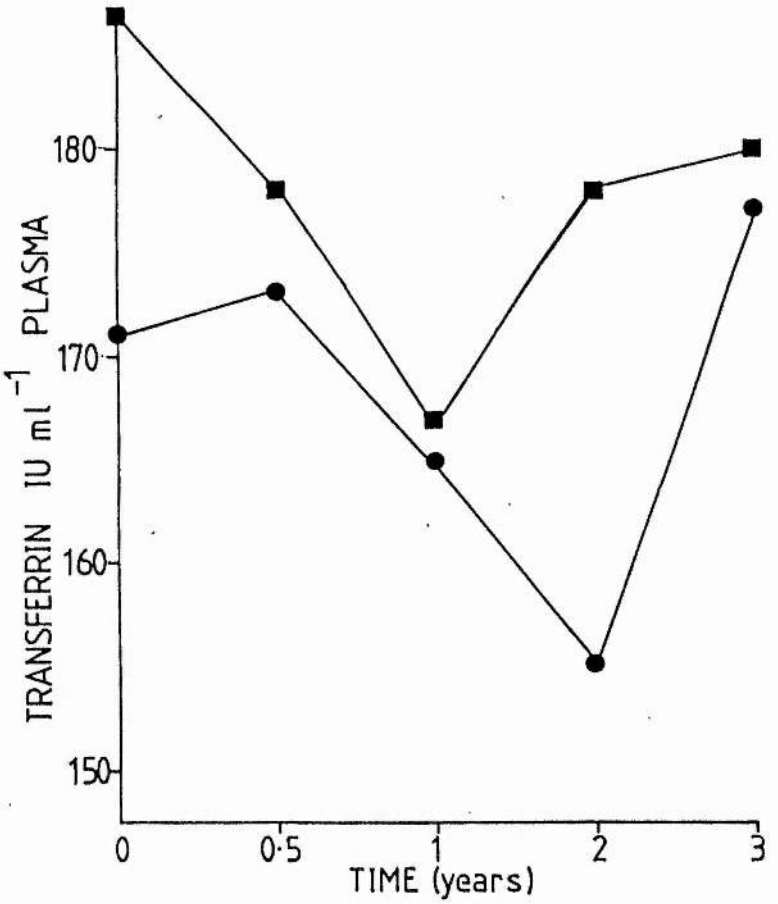


Figure 64. Transferrin concentration in plasma after various times of storage at -20°C measured by radial immunodiffusion. Each point represents the mean for each group of subjects and assay were carried out in duplicate estimations. ●—●, control; ■—■, DMD.

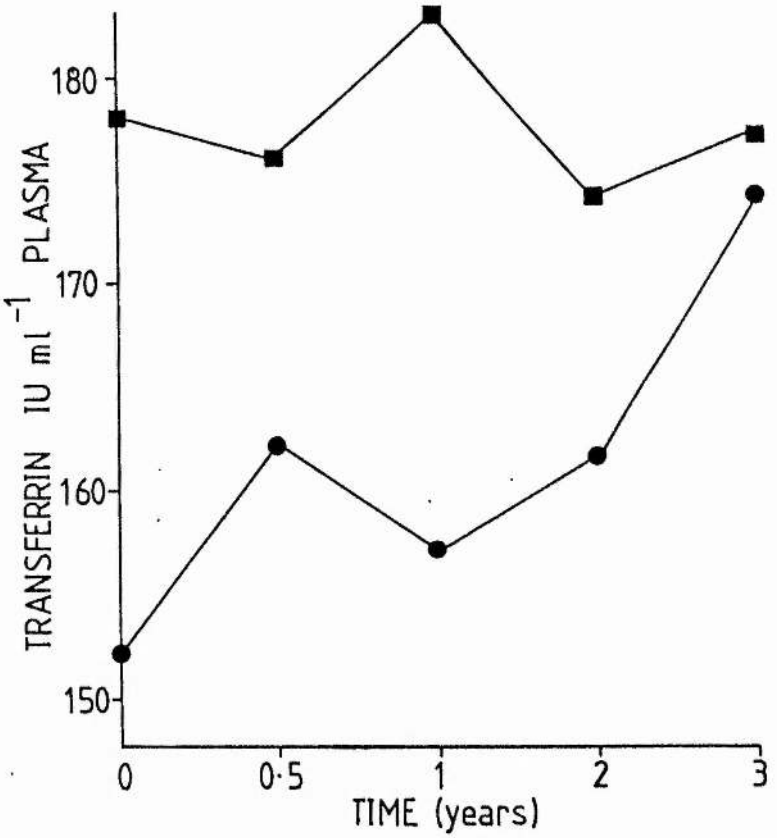


Figure 65. Transferrin concentration in plasma after various times of storage at -20°C measured by rocket immunoelectrophoresis. Each point represents the mean for each group of subjects and assay were carried out in duplicate estimations. ●——●, control; ■——■, DMD.

4 DISCUSSION

LIPID PEROXIDATION IN CULTURED SKIN FIBROBLASTS

The aetiology of DMD remains unknown at this stage. A number of theories which have been prominent in the last decade including the neurogenic and the vascular thoeries, appear unlikely to be true (Bradley et al.,1975). A search continues for the underlying biochemical defect, and in recent years membrane abnormalities (Membrane theory) both in skeletal muscle and in other cells such as the RBC, lymphocyte, plaletet have been reported (see Rowland, 1980).

In addition CSFs have also been used by a few laboratories as model systems in the search for a membrane defect, specifically associated with membrane lipids. CSFs from DMD patients were characterized by histological and morphological techniques (Wyatt and Cox, 1977). These studies revealed increased numbers of lipid storage bodies and lysosomal bodies in DMD CSF lines. They also found the consistent appearance of a specific abnormal cytoplasmic body, which appeared in DMD and not in any normal cells or those from other genetic diseases. However, this has since been refuted by other workers (Roses et al.,1980 and Rowland, 1980) whose studies on CSF morphology have been unable to differentiate DMD and normal control CSFs using phase-contrast light microscopy, histochemical stains, and transmission electron microscopy. In contrast, they have found

inclusions similar to those reported by Wyatt and Cox (1977) in both normal control and dystrophic cell lines. Cullen and Parson (1977) also reported no morphological differences using electron microscopy.

Other experiments could be called biochemical ones. With the development of lipid analytical techniques - thin layer chromatography and gas-liquid chromatography - the first reliable results concerning lipid composition in CSFs from DMD lines were published. One of the first studies of phospholipids and fatty acid composition was done by Kohlschutter et al (1976), who found no abnormality in the composition of phospholipids and fatty acids in either young or old cultures of dystrophic CSFs. However, there are some limitations to Kohlschutter's work in that separation of phospholipid classes PI and PS was not achieved and so these were considered together. This does not give any information about each as an individual phospholipid. More importantly, plasmalogens were not investigated which are found in almost every tissue in the body and are especially abundant in the more excitable tissues like muscle and brain. This fraction may be as much as 12% of total phospholipid fraction. Regarding fatty acid classes, only seven were identified with a possible 6-8% of fatty acids (minor components) unidentified. In the other lipid study Kunze and Sperling (see Kunze, 1977), based on only three cases, found that only the choline plasmalogens - measured as a reduction in dimethylacetal - were diminished. All other classes showed no abnormalities.

However, in this investigation, CSFs were chosen as a model system to investigate the possibility that pathological alterations in membrane integrity could be caused by free radical lipid peroxidation. It was found that in both normal and DMD CSF cultures lipid peroxidation increased progressively as incubation times increased (Figs. 21-24 and Tables 6-7). This agrees very well with the studies of lipid peroxidation in CSFs from normal cell lines carried out by Gavino et al (1981) who also showed that MDA production was time-dependent and that addition of PUFA to experimental medium produced a significant increase in lipid peroxidation: MDA was increased significantly in the presence of 8,11,14-eicosatrienoic acid, 5,8,11,14-eicosatetraenoic acid, and 7,10,13,16-docosatetraenoic acid. The present studies support these findings (Figs. 23-24 and Table 7). It was found that 5,8,11,14-eicosatetraenoic acid (arachidonate) generated significantly increased MDA in a time-dependent manner when added to cultures of CSFs. Comparing normal and DMD lines (Figs. 21-24 and Tables 6-7), our results certainly shown that MDA production in DMD CSFs exceeds that in the normal by almost 8 times (depending on conditions). The eventual decline of MDA production (i.e. 72 hours up to 96 hours of incubation) may be due to the lack of additional peroxidisable PUFA, to the reaction of the MDA with amino groups of amino acids, proteins and amino-phospholipids, to the further oxidative decomposition of MDA itself and to condensation reactions in which MDA forms unreactive polymers.

A comparison of unwashed (Figs. 21 and 23) with washed

data (Figs. 22 and 24) revealed a marked difference between the two values. The possible cause for the difference between washed and unwashed CSFs is difficult to explain. It is possible that some extracellular antioxidant in the medium or maybe antioxidant factors, loosely attached to CSF cell membranes, were removed together with the medium during the washing stage and render the washed CSFs more susceptible to peroxidative damage which is ultimately responsible for the difference between their MDA data and those of unwashed CSFs. An alternative explanation is that the decrease in concentration of MDA in unwashed CSFs is due to the presence of culture medium, which might in some way interfere with the reaction between TBA and MDA or MDA precursors.

A significantly greater amount of MDA was formed when human CSFs from both patients with DMD and normal were grown in experimental medium with PUFA (Figs. 23-24 and Table 7) compared with those grown without PUFA (Figs. 21-22 and Table 6). The PUFA (20:4) used here could be source of oxidant stress in both DMD and control CSFs, although the effect is more prominent in DMD as shown by higher TBA-reactive materials in DMD CSFs (Table 7 and Figs. 23-24). According to Gavino and co-workers (1981) the oxidant stress of PUFA arises through the action of lipoxygenase, cyclooxygenase or by oxidation by the microsomal NADPH oxidation system. As a result of the former two enzyme activities, CSFs treated with PUFA synthesize large amounts of prostaglandin metabolites as well as MDA, therefore a component of the lipid peroxidation which occurs in CSFs is enzymic. It should be possible to eliminate the contribution of prostanoid

biosynthesis to the overall yield of lipid peroxidation products by using cyclooxygenase inhibits such as indomethacin (Gavino et al.,1981).

Interestingly, in the presence of 20:4, DMD but not normal CSF showed measurable MDA at 0 time (in washed CSFs, Fig. 24), which emphasized the difference between DMD and normal CSFs.

Cells treated with the PUFA show large increases in the number of lipid droplets (TG accumulation), lysosomal enzyme activity (Miller et al.,1980) and synthesis of PGE_1 and PGE_2 (Huttner et al.,1977; Cornwell et al.,1979). These PUFA metabolites inhibit the extent of cell proliferation (Bockman and Rothschild, 1979). Alpha-tocopherol (vitamin E) restores cell proliferation even though it has no effect on TG accumulation, lipid droplets, lysosomal enzyme activity (Miller, 1980) and prostaglandin biosynthesis (Cornwell et al.,1979). Since alpha-tocopherol is an antioxidant, it could restore cell proliferation through the inhibition of lipid peroxidation (Gavino et al.,1981).

With regard to cells treated with TBH (see section 3.1.1.5), a very high concentration (300µm) not only induces lipid peroxidation but most importantly leads to rapid cell damage as shown by detachment from the substratum (in both DMD and normal CSFs). Attachment of CSFs is a process that depends on several surface-associated protein or other molecules, which may be denatured by the action of TBH directly on the effect

might be due to killing of the cells and generalised membrane disruption (i.e. the attachment points are left behind). Therefore it will be of interest to see whether DMD CSFs behave differently from that of control CSFs when treated with the concentrations of TBH (50μm) determined in this work to have no cytotoxic effects to the CSFs (see section 3.1.1.5).

In conclusion the results albeit for a small number of individual cell lines, show that, whether induced by added PUFA or endogenous, the amount of lipid peroxidation occurring in DMD CSFs in culture was significantly and consistently greater than in normal cells at all times of incubation. It is clearly of great importance to confirm these findings with larger numbers of cell lines. The reason for this difference between DMD and normal CSFs should also be further investigated. It is unlikely to be due to decreased activity of the recognised antioxidant enzymes, SOD, CAT, GSHPx or GSHR as these have all been shown to be normal (Hunter and Amin, 1984). However increased lipid peroxidation might be due to a deficiency in some untested antioxidant (vitamin E, GSH?) or to increased enzymic or non-enzymic production of free radicals.

LIPID PEROXIDATION IN PARTICULATE FRACTION FROM CSFs

To determine whether the increased lipid peroxidation in DMD CSFs was a property of membranes/organelles alone or dependent on stimulatory cytosolic factors, the particulate fraction for CSFs was prepared and incubated with NADPH (the enzymic system). As expected, NADPH significantly stimulated lipid peroxidation in both DMD and normal control fractions as monitored by both MDA (Fig. 25) and FP (Fig. 26). Although levels of FP were somewhat greater in the DMD fraction than in the normal control (Table 8 and Fig. 26), there was no dramatic difference as found for whole cultures. However these negative results do not exclude the possibility that lipid peroxidation is increased in DMD whole CSFs. There are at least three possible reasons for these negative results.

The number of CSFs lines available for experimentation were small in both DMD (n=1) and normal control cells (n=1). The use of more CSF lines might show significant differences.

Secondly, CSF membrane lipid peroxidation may not be entirely a membrane-dependent process. It is likely that other factors than CSFs membrane components could be involved such as cytoplasmic components like Fe^{+3}, and reducing agents such as ascorbate. It is known that rates of lipid peroxidation, *in vitro* are extremely low unless transition metal ions are present (Fujimoto *et al.*,1984), iron being especially effective in the

presence of a reducing agent, e.g. ascorbate (Pryor, 1966). Both the reduced and oxidised metal ions were effective catalysts (Gutteridge et al.,1977). The reaction between O_2^{-o} and H_2O_2 to give ^{o}OH does not occur unless traces of iron salts are present in the system ((Rowley and Halliwell, 1983). In addition, copper can cause autoxidative rancidity in dairy products as well as in human tissues (Rowley and Halliwell, 1983), and iron catalyzes the decomposition of hydroperoxides as follows.

$$Fe^{+2} + ROOH \longrightarrow Fe^{+3} + RO^{o} + OH^{-}$$

$$\underline{Fe^{+3} + ROOH \longrightarrow Fe^{+2} + ROO^{o} + H^{+}}$$

$$2ROOH \longrightarrow RO^{o} + ROO^{o} + H_2O$$

Lipid peroxidation is well known to be catalyzed by haemoglobin, iron protoporphyrin, and iron salts. As has been pointed out by Eriksson et al (1971) the iron porphyrin groups of haemoproteins are active in both enzymic and in non-enzymic lipid peroxidation. It appears that this type of catalysis is very important in peroxidation that occurs in biological systems. Fujita (1972) has studied the interrelationships between lipid peroxidation, lipid composition, and cytochrome oxidase activity in rat liver mitochondria. Metal ions (Fe^{+2}, Fe^{+3}, and Cu^{+2}) have been found to catalyze lipid peroxidation by increasing the formation of lipid peroxides. These ions also decrease cytochrome oxidase activity and the unsaturated fatty acid content of the mitochondrial membrane. Total sulfhydryl content of liver microsomes and of the soluble fraction are also decreased by ferrous iron (Fujita, 1974). In addition Wills

(1972) has shown nonhaem iron to increase the formation of lipid peroxides in rat liver microsomes (14% increase). This results in a partial disintegration of SR membranes and loss of various membrane-related functions such as oxidative demethylation (Chiva and Mato, 1984). Wills postulates that a small portion of the injected iron is converted into an electron transport component which increases the rate of NADPH-induced lipid peroxidation. The subcellular distribution of metal ions includes the cytosol (Witting and Steffen, 1984) and membrane fraction (Barber and Berheim, 1967). In these experiments with CSF particulate fraction, the ability of these various metal ions (Fujimoto et al.,1984) and ascorbate (Pryor, 1966; Vladimiro et al.,1980) to initiate and propagate free-radical-mediated lipid peroxidation in CSFs membrane will be reduced, since a considerable amount may be lost during centrifugation and washing procedures.

Another possible explanation for these results may be that lipid peroxidation products (if already formed) from CSF membranes do not remain in the membrane but may be released into the cytoplasmic fraction. Thus, as already mentioned above for initiators, the products may also be lost during washing procedures. As a result the total amount of peroxidisable material may be decreased during preparative and washing procedures in both DMD and control fractions.

Lastly, one further possible explanation might be the presence, in the cytoplasm of normal CSFs of a greater amount/activity of some protective factor, hitherto

uninvestigated.

Therefore, failure to find and characterize a significant difference in enzymic lipid peroxidation in DMD and normal control CSF membranes does not preclude the existence of such a difference in CSFs as a whole. To attempt to further clarify this question, whole homogenates of CSFs were examined for enzymic and non-enzymic lipid peroxidation.

LIPID PEROXIDATION IN CSF TOTAL HOMOGENATES

Non-enzymic and enzymic (Table 9) *in vitro* peroxidation of total CSF homogenates revealed a difference between DMD and normal controls as measured by CD (Figs. 27-28) and FP (Figs. 31-32) but not by MDA (Figs. 29-30). The relatively small increase of lipid-soluble fluorescence components over the time periods of incubation used, for both the enzymic and non-enzymic systems, along with the relatively high values for CD suggest that in CSF homogenates lipid peroxidation is still at a relatively early stage of the total process. Lipid peroxidation can be divided chronologically into the following phases: initiation, where a hydrogen atom is abstracted from an unsaturated fatty acid (PUFAH), by an initiating free radical (R°) and a lipid conjugated diene free radical is formed ($PUFA^{\circ}$); propagation, where the free radicals interact with molecular oxygen to form peroxyl radicals ($PUFAOO^{\circ}$) that then act as initiators, abstracting further hydrogen to form hydroperoxides (PUFAOOH) which in turn can decompose to form peroxyl or alkoxyl ($PUFAO^{\circ}$) free radicals; and chain termination, where two radicals meet and end the propagation or where an antioxidant, e.g. alpha-tocopherol, provides a hydrogen atom to block the free radical abstraction from another PUFAH. From the hydroperoxides, additional decomposition carbonyl products are formed including aldehydes and ketones. MDA is one of the several carbonyls formed (Dahle *et al.*,1962). MDA was shown to react with amino compounds by Crawford *et al* (1967). Further, Chio and Tappel

(1969) have shown that the reaction of MDA with amines forms a fluorescent chromophore characterized as N,N disubstituted 1-amino-3-imino-propane, R-N=CH-CH=CH-NH-R', having an excitation maximum at 370-400nm and fluorescence maximum at 450-470nm. In addition, during *in vitro* peroxidation of subcellular organelles, similar FP were formed (Chio *et al.*,1969).

Given the sequence of this complex series of reactions, it is at first sight surprising that whilst differences between CD and FP are discernable between DMD and normal CSFs, no difference in MDA evolution was found. There are at least three possible explanations for this. Firstly, FP estimation is acknowledged to be a method giving several orders of magnitude greater sensitivity then MDA estimation (Logani and Davies, 1980) so that the latter may not be sufficiently sensitive and precise to pick up relatively small changes. Secondly, it has become apparent that MDA may not be the major carbonyl compound in a complex mixture of carbonyls produced by peroxidising biological systems. Furthermore it is likely that the relative amounts of these carbonyl compounds are highly dependent upon the particular membrane system involved (Esterbauer *et al.*,1982). Therefore it is possible that the CSF system, which has not been hitherto examined in any detail, may produce rather small amounts of MDA but that other carbonyl compounds may well be of equal or greater importance in the production of cellular pathology (Witting, 1980; Slater, 1972) and FPs. Finally Ghoshal and Recknagel (1965) have suggested that a reduction in or the absence of MDA from a peroxidising biological system may be the result of its

rapid metabolism.

As a summary, the three series of experiments described here which examined lipid peroxidation in: (i) whole cultures; (ii) isolated washed particulate fraction and (iii) total CSF homogenates produced interesting yet puzzling results. The greatest difference between normal and DMD CSFs was seen in whole cultures where, as judged by MDA, there was a marked increase in lipid peroxidation in DMD. CSF homogenates showed significant but smaller increase in lipid peroxidation in DMD preparations under conditions where lipid peroxidation was induced enzymically or non-enzymically, but only when monitored by CD and FP, not by MDA. This may suggest that there is a potent stress factor in confluent cultures which induces increased lipid peroxidation, particularly in DMD cells. The difference between DMD and normal cells was not apparent in isolated washed particulate fractions where lipid peroxidation was induced enzymically which suggests that either a soluble potentiating factor for lipid peroxidation is present in greater amounts, or that a hitherto uninvestigated soluble protective factor is diminished, in DMD CSFs.

ASSAY OF TOTAL ANTIOXIDANT ACTIVITY OF CSF CYTOSOL USING RAT LIVER PARTICULATE FRACTION AS A MODEL SYSTEM

The microsomal membrane has been used by many laboratories as a model membrane in studying lipid peroxidation (Pederson and Aust, 1975; Koster and Slee, 1980; Lokesh et al.,1981; Cheeseman, 1982). It is known to be very susceptible to lipid peroxidation due to its high content of PUFA (Koster and Slee, 1980). In fact, mitochondrial membranes have been shown to peroxidize at three times, and those of microsomes at ten times, the rate of lysosomal membranes (Desai et al.,1963). Based on this information, total particulate fraction from rat liver was chosen as a model system to evaluate the AOA of CSF cytosol. Both classes (non-enzymic and enzymic) of induced lipid peroxidation processes were used in this study. The non-enzymic process was induced by iron/ascorbate while the enzymic processes was NADPH-dependent although the presence of ADP-chelated iron (Hochstein and Ernster, 1963) is also needed. Iron has been found to be a vital component of cytochrome-P_{450}, an oxidase involved in the electron transfer system associated with lipid peroxidation (DeMatteis and Sparks, 1973).

In the pilot studies to evaluate the rat liver system, the formation of lipid peroxidation products was shown here, as expected, to be a time-dependent process (Table 10). In both cases (enzymic and non-enzymic systems), the values increased rapidly up to a maximum at sixty minutes of incubation, except FP

which peaked at 120 minutes (Figs. 35 and 38). As predicted, thereafter any further incubation resulted in a sharp decrease in CD (Figs. 33 and 36) and MDA (Figs. 34 and 37). A possible explanation for the reduction of the peroxidation products (CD and MDA) at this stage may be that most of the membrane PUFA had been consumed. Arachidonic acid contained in the microsomal choline and ethanolamine phosphoglyceride is probably the main substrate for microsomal lipid peroxidation (Lokesh et al.,1981) and a decrease in peroxidation activity is associated with a reduction in the PUFA content of the microsomal phospholipid, particularly arachidonic acid (Lokesh et al.,1981).

It is consistent with the chemistry of lipid peroxidation that FP (Figs. 35 and 38) as already mentioned above, continuously increased beyond sixty minutes reaching a maximum at 120 minutes which was sustained for the remaining 60 minutes of incubation. Also consistent is the decrease of MDA concentration beyond sixty minutes. Shimasaki et al (1977) studied the FP that developed during the oxidation of unsaturated lipids in an aqueous emulsion with glycine. Formation of these FP correlated with the decrease in CD and TBA-reactive materials. Thus these observations are consistent with their findings and the known chemistry of lipid peroxidation (see introduction and Fig. 3).

Under normal physiological conditions living cells possess adequate defence mechanisms against lipid peroxidation. Vitamin E is of undoubted importance as a general free radical scavenger and antioxidant in the lipid phase of membranes and some

consequences of vitamin E deficiency have been reported (Bieri et al.,1983). The possible involvement of SOD and CAT as scavengers of O_2^{-o} and H_2O_2, respectively, in the inhibition of NADPH-induced lipid peroxidation has been challenged (Pederson and Aust, 1975), particularly in the case of CAT which is largely confined to the peroxisomes of most cell types (RBC being a notable exception). The other enzymes which are important antioxidants are the GSH-dependent enzymes.

It has been generally accepted that protection against the oxidative damage of cell membranes is partially, at any rate, mediated by the reduction of endogeneously formed lipid hydroperoxides to hydroxy derivatives through GSHPx. However, McCay et al (1981) and Burk et al (1980) suggested that an additional rat liver cytosolic factor, which protects against peroxidation of microsomal lipids in the presence of GSH, is not GSHPx. The former group showed that peroxides of microsomal lipids were very poor substrates for GSHPx activity and suggested that the protection was conferred by prevention of peroxidation rather than by reducing lipid hydroperoxides once formed. Levender (1982) found that GSHPx appears unable to utilize PUFAOOH (fatty acid hydroperoxides) esterified in membrane phospholipids as a substrate even though it acts effectively on fatty acid salts and free fatty acid peroxide (McCay et al.,1981). For efficient detoxification of PUFAOOH by GSHPx its therefore necessary for the prior release of PUFAOOH by

phospholipase A. Therefore, McCay et al (1981) suggested that the most crucial intracellular antioxidant defences are GSH-dependent factors. Chromatographic studies by Burk et al (1980) suggested that the protective effect of GSH against formation of MDA in rat liver preparations may be mediated by GSH-S-transferrase (EC 2.5.1.18), which cannot utilize H_2O_2. On the contrary, Ursini et al (1982) and Mariorino et al (1982) purified a cytosolic inhibitory protein from pig liver and heart and found it to be distinct from both known GSHPx and GSH-S-transferrases in agreement with McCay et al (1980, 1981).

In the experiments described here, CSF cytosol from both DMD and normal controls inhibited both enzymic (Fe^{3+}/ADP/NADPH) and non-enzymic (ascorbate/Fe^{3+})-stimulated lipid peroxidation in rat liver particulate fraction but to a different extent (Figs. 39-44). It was found that DMD CSF cytosol was more effective in inhibiting the formation of peroxidation products in both systems, and as judged by all three indices of lipid peroxidation, although the effect was more pronounced for CD (Figs. 39 and 42) and FP (Figs. 41 and 44) measurements than for MDA (Figs. 40 and 43). Again the significance of these results is perhaps questionable due to the small number of cell lines tested. However, since it has been shown (Hunter and Amin, 1984) that SOD, CAT, GSHPx and GSHR are normal in DMD CSFs, this would infer that some other, GSH-dependent factor may be increased in DMD CSFs. However the posibility that CSFs cytosol induces or potentiates lipid peroxidation in addition to inhibition was not fully investigated in this work.

These results are at first sight paradoxical since DMD CSFs appear to have a better protection system than normal yet show a greater propensity for lipid peroxidation. However, the raised levels of AOA may reflect an attempt by DMD CSFs to compensate for the increased lipid peroxidation as suggested by Kar and Pearson (1979) for DMD muscle. But it is clearly not completely successful presumably because it cannot cope with the excess and rapid production of peroxidation products.

LIPID PEROXIDATION PRODUCTS IN FRESH PLASMA

The three methods most widely used for measurement of lipid peroxidation are U.V absorption (CD), TBA-assay (MDA) and fluorimetric technique (FP). Using these methods this study found significantly increased plasma lipid peroxidation products (CD,MDA and FP) in DMD compared with normal control plasma samples (Figs. 45-47). Levels of CD were raised by 77% ($P<0.02$), MDA by 35% ($P<0.01$) and FP by 70% ($P<0.001$).

Increase in lipid peroxidation products in DMD plasma might be explained by either one or a combination of the following factors: (i) an enhancement of platelet aggregation which was reported in DMD (Yarom et al.,1983) may be associated with an activation of platelet thromboxane synthesis and prostaglandin release, reported by others as major sources of MDA in plasma (Szeceklik et al.,1981); (ii) Increased lipid peroxidation in muscles. Since morphological study of DMD muscle reveals varying degrees of degenerative changes in most of the muscle fibres (see introduction), associated with significantly higher TBA-reactive materials (Kar and Pearson, 1979; Mechler et al.,1984), it may be reasonable to assume that these changes could be responsible for the leakage into blood of muscle lipid peroxidation products in the same way as muscle enzymes, such as CPK. (iii) Release of substrates and peroxidation catalysts for autoxidation. Since Fe^{2+} is such an important and potent catalyst for lipid peroxidation in biological systems (Halliwell

and Gutteridge, 1984), it is also possible that the extensive muscle cell damage and necrosis in DMD releases Fe^{2+} (and possibly membrane fragments and lipids) into the circulation and that the lipid peroxidation products are then generated *in situ* in the circulation from e.g. lipids in association with plasma lipoproteins. It has been shown that lipid peroxides (Szeceklik *et al.*,1981) and the breakdown product of lipid peroxidation, MDA (Fogelman *et al.*,1980) can be transported by LDL.

Proteins are powerful free-radical scavengers (i.e. potential antioxidants); and one must assume that in normal cells they help to insulate autoxidisable lipids and lipid peroxidation catalysts (i.e. Fe^{+3}, Fe^{+2}, and Cu^{+2}) from active oxygen radicals (Gutteridge and Halliwell, 1984). No free metal ions are found in human plasma but traces of metal ions are present (see Rowley and Halliwell, 1983) intracellularly and in several other body fluids other than plasma. In plasma, copper ions exist bound to albumin and to amino acids such as histidine (Rowley and Halliwell, 1983) while iron is bound to the abundant iron-binding protein, transferrin, (Gutteridge *et al.*,1982). Free iron is only detected in the serum from patients suffering from iron-overload diseases (Hershko *et al.*,1978).

However, this structural insulation could be damaged in some types of disease such as DMD. Proteins as well as membrane lipids are susceptible to free-radical damage as suggested by Gutteridge (1982). Further, both free iron (Gutteridge *et al.*,1982) and copper react would readily and rapidly with H_2O_2 to

give $O_2^{-\cdot}$-dependent and ascorbate-dependent $^{\cdot}OH$ formation in such pathological plasma.

In this study a comparison between plasma lipid peroxidation products in adults and children revealed no significant difference except for MDA which was increased 20% in adults compared with children ($P<0.05$) (Fig. 49). Increased MDA is expected in adults since Poubelle et al (1982) showed there is a linear increase in plasma TBA-reactive material with age, in normal healthy individuals. Paradoxically, at least at first sight, there is no increase with age of subject in the two other lipid peroxidation indices, CD and FP. There may be at least two interpretations for this data. Firstly, it is likely that the reactions from CD to yield MDA are rapid and so CD levels never build up but assume a relatively constant steady-state level. Lipid soluble FP in plasma are likely to be transported in association with lipoproteins and, as such, to be turned over and removed from the circulation. Levels of FP in plasma then may well be transient although in most cells, because of their localisation in lysosomes, FP accumulate and probably give a measure of the cumulative lipid peroxidation which has occurred in the cell's life time. To account for increased FP in DMD plasma as compared to controls, the situation in DMD may be different in that much higher levels of lipid peroxidation products may be produced, perhaps in bursts corresponding to damage and release from muscle. Secondly, the methods (CD and FP, or may be MDA?) may be unreliable indicators of peroxidative damage in vivo at least in plasma.

EFFECT OF STORAGE ON PLASMA LIPID PEROXIDATION PRODUCTS

This study has shown that when normal plasma is stored for between six month and three years, of the lipid peroxidation products, MDA is increased two fold (Fig. 52), FP three fold (Fig. 53) but there is no change in CD (Fig. 51). Similar changes were found for DMD plasma except that there was a slight decrease in CD, in addition. The absolute level of these peroxidation products (CD,MDA and FP) was always higher in DMD plasma than in normal control throughout the time of storage (Table 15).

CD concentration (A_{235}) appears not to be significantly correlated with storage time either in normal controls or in DMD plasma samples. CD is a relatively unstable fatty acid radical, which is readily further metabolized or reacts with molecular oxygen to form, ultimately, the potentially more damaging peroxidation product, MDA. CD, therefore is a transient intermediate whose steady state concentration is likely to be low. MDA, is a bifunctional cross-linking agent (Wold, 1972) which may form condensation products with plasma proteins, lipids or lipoproteins and yield intra- or intermolecular cross-linking (Chio and Tappel, 1969). Using SDS-polyacrylamide gel electrophoresis, Janado et al (1979) recently demonstrated that MDA could cause the formation of oligomers of bovine serum albumin.

From the results presented here, the lipid peroxidation reactions occurring at -20°C can be divided into an initial slow rate which occurs in the first year which is followed by a very rapid oxidation rate thereafter. The "lag phase" may be due to, e.g. consumption or inactivation of endogenous antioxidant(s) or to the exponential build-up of free radicals. It is noteworthy that the kinetics of free radical chain reactions normally follow an exponential course and the low temperatures of storage may simply serve to accentuate this initial phase. Swartz (1971) has shown that the damage which occurred during the freezing of bacteria (E.coli) was related to oxygen-free radical formation. Later, Swartz (1973) reviewed the concept that oxygen toxicity effects include autoxidative damage to cells which occurs during freezing in the presence of molecular oxygen. In addition, Finean (1954) studied the effect of freezing on normal nerve and found damage associated with denaturation of the protein component (maybe via MDA cross-linking?) with consequent breaking of links between lipid and protein, this in turn resulting in a breakdown of some of the lipoprotein complexes. It is probable that the same process has occurred in plasma during storage where as a result of damage to plasma lipoprotein peroxidizable free fatty acids (i.e. unbound to plasma protein) and free lipids (not necessarily phospholipids) would be released to react with molecular oxygen in the frozen aqueous phase. Phospholipid is not the only peroxidizable lipid in plasma, since fractionation studies by Thayer (1984) on serum lipid peroxides in rats demonstrated that several distinct types of lipid peroxides were present within the major neutral lipids, including TGs,

cholesterol ester and free cholesterol. Interestingly, the serum phospholipids did not appear to contain comparable amounts of peroxidized lipids (Thayer, 1984). In this respect, it would be desirable to repeat the plasma storage experiment described here and to fractionate the lipids especially to determine the cellular origin of plasma lipid peroxides as well as its relation to the available neutral lipids in plasma.

To establish whether lipoprotein other than LDL provide lipid or phospholipids for MDA formation in plasma, Alaupovic et al (1972) tested very low density lipoproteins (VLDL), high density lipoproteins (HDL), and subfractions of low- and high-density lipoproteins, originally isolated in the presence of 0.05% EDTA from fresh plasma and outdated plasma (Alaupovic et al.,1972). All lipoprotein samples showed increased MDA concentration upon storage irrespective of their density properties and regardless of whether they were originally isolated from fresh or stored plasma. In contrast, two LDL samples isolated from fresh plasma in the presence of EDTA, glutathione and N_2 gave negative reactions with TBA following storage for 14 days in N_2 at $4^{\circ}C$. These results suggest that lipids in all lipoproteins are susceptible to peroxidation (Lee, 1980), provided molecular oxygen is present.

Lipid peroxidation affects not only the solubility and structure of plasma components but also their uptake by cells. Fogelman et al (1980) demonstrated that the uptake of the MDA-modified LDL by human monocyte-macrophages were significantly

different. Lee (1980) therefore suggest that plasma protein and lipoprotein to be used for metabolic or structural studies ought to be isolated under oxidation-free conditions.

To investigate the effects of storage over a shorter time scale, lipid peroxidation products from two pools of plasma of two normal adult individuals (A and B) revealed that both samples were rather stable over the first month (Fig. 54), but that thereafter there was a linear increase in both MDA and FP (Table 16 and 54) up to six months of storage. More importantly the rate of lipid peroxidation varied from individual to individiual. These variations in the rate of lipid peroxidation product formation may be a reflection of both their plasma antioxidant contents, e.g. vitamin E and carotenoids, and their PUFA contents, particularly those containing methylene-interrupted double bonds.

TOTAL ANTIOXIDANT ACTIVITY IN PLASMA

The findings reported by Barber (1961) who suggested that serum has a powerful antioxidant effect prompted others (Vidlakova et al.,1972) and confirmed their works. Recently Dormandy et al (1974) suggested that variations in serum antioxidant activity (AOA) may have considerable clinical relevance. Based on these findings, Stocks et al (1974) saw the need for, and created, a standard biological antioxidant assay, i.e. an autoxidizing system against which the inhibitory effect of serum and of other biological fluids can be measured. Fresh ox-brain homogenate prepared under standard conditions at $4^{\circ}C$ and stored at $-20^{\circ}C$ has been found to autoxidize spontaneously and reproducibly when reheated to $37^{\circ}C$. In this study it was found that lipid peroxidation was inhibited by DMD plasma by a mean of 76% compared with 63% for normal plasma (Table 17). The differences between DMD and normal controls are highly significant ($P<0.001$; Fig. 55). Also there was a strong positive correlation ($P<0.05$) between AOA and MDA (Table 18) in DMD but not control plasma suggesting that increased AOA activity may result from increased lipid peroxidation, in DMD.

VITAMIN E (ALPHA-TOCOPHEROL) ANALYSIS

The presence of circulating vitamin E may help to protect the heart against hypoxic trauma (Guarnieri et al.,1978). But Jackson et al (1983) reported that this does not appear to be true for exercise-induced damage to skeletal muscle, since adding alpha-tocopherol to the incubating medium did not influence the release of lactate dehydrogenase from mouse soleus muscles in vitro. Plasma vitamin E concentrations were reported to be normal in patients with DMD (Jackson, 1983, unpublished results). However, significantly decreased ($P<0.002$) alpha-tocopherol concentrations were found in DMD plasma compared with normal controls in this work (Fig. 56). The discrepancy in these findings is difficult to explain. Since circulating vitamin E will partition with the hydrophobic region of the membranes of tissues, this finding raises the possibility that tissue (and particularly muscle) vitamin E may also be reduced in DMD. No significant correlation was found between vitamin E and AOA ($P>0.05$; Table 22). Therefore, this suggests that the role of vitamin E as an antioxidant in plasma is minimal. Recent work suggest that the AOA of plasma largely depends not on vitamin E (Vidlakova et al.,1972) but on a plasma protein fraction (Gutteridge and Stocks, 1981). The finding that plasma AOA is destroyed by heating and is non-dialysable indicates that it is due to plasma proteins. However, it is not a non-specific function of plasma proteins. The two antioxidant protein fractions isolated from plasma together comprise approximately 4%

and have been identified as the beta-globulin, transferrin and the alpha$_2$-globulin caeruloplasmin (Gutteridge and Stocks, 1981) (see below). Further, in agreement with the findings of this work the experiments described by Stocks et al (1974) provide strong evidence that the contribution of vitamin E to the AOA of normal adult human serum is negligible. When tested against brain homogenate whole serum is approximately 50 times more powerful as an autoxidation inhibitor than can be accounted for by its tocopherol content. But caeruloplasmin and transferrin, (Al-Timimi and Dormandy, 1977) inhibited ox-brain homogenate lipid peroxidation at concentration at which they occurs in plasma.

Since the requirement for vitamin E in animals and man (Century et al.,1961) has been related to the total amount and composition of lipid, particularly to PUFA, either ingested or accumulated in tissues or both, measurement of vitamin E concentrations alone may not give complete information regarding its role as a naturally occurring antioxidant. Indeed many other workers have suggested that plasma vitamin E (bound almost exclusively to beta-lipoprotein, (Davies et al.,1969)) levels are strongly correlated with total plasma lipid concentrations (Bieri and Farrell, 1976; Farrell, 1980), and that since concentrations of human plasma lipid can show marked variations, Burton et al (1982) suggested that in order to compare plasma vitamin E levels, they should be related to lipid levels in plasma, a point which has been emphasized by others (Bieri and Farrell, 1976; Farrell, 1980). This was not done in the work described here but

should be carried out before attempting to assign pathological significance to the reduced plasma vitamin E in DMD patients.

Peroxidation of tissue lipids may be the primary damage in vitamin E-deficiency which then causes the spectrum of metabolic derangements observed in vitamin E-deficiency diseases. This topic has been reviewed by Tappel (1962). Many of the vitamin E-deficiency symptoms seen in experimental animals are histologically degenerative and result in tissue wasting. Vitamin E deficiency is a well-recognized cause of myopathy in many animal species and has been claimed by some authors to provide a good model of the human muscular dystrophies (Kakulus, 1969) but the mechanism of the damage is obscure (Jackson et al.,1983). One of the primary function of vitamin E is to inhibit unsaturated lipid peroxidation in the tissues of animals, membranes containing the highly unsaturated fatty acids being particularly vulnerable (Tappel, 1962).

Heavy exercise may induce necrotic myopathy in skeletal muscle (Vihko et al.,1978) and is reported to cause increased lipid peroxidation, both in animals (Gee and Tappel, 1981) and in humans (Dillard et al.,1978), and animals with vitamin E-deficiency (Jackson et al.,1983).

It was suggested that in nutritional muscular dystrophy the first consequence of vitamin E-deficiency may be free-radical damage to cell membranes, including those of lysosomes (Zalkin and Tappel, 1959). Both mitochondria and microsomes also showed

evidence of lipid peroxidation (Tappel and Zalkin, 1962). Lipid peroxidation results in fragility and rupture of lysosomes with the release of catabolic enzymes (Tappel, 1962). Indeed, the lysosomal enzymes (beta-glucoronidase, beta-galactosidase, cathepsin, aryl sulfatase, acid phosphatase, and acid ribonuclease) in leg muscle of vitamin E-deficient rabbit were increased before the histopathological signs of muscular dystrophy (Zalkin et al.,1962). Cell death may be caused by the catabolic action of enzymes releases from ruptured lysosomes and from effects of generalized membrane damage. It is now well documented that loss of proteins from human dystrophic muscle results primarily from an increased rate of protein catabolism (McKeran, 1977) by both lysosomal and non-lysosomal enzymes (e.g proteinases) (Pennington, 1977; Kar and Pearson, 1980). A majority of these proteinases have been found to be increased in human muscular dystrophy and other diseased muscles (Kar and Pearson, 1980). Invasion of injured tissue by macrophages and phagocytic leukocytes is known to occur following many causes of cell injury.

Macrophages are particularly rich sources of lysosomal enzymes and their presence may be sufficient to account for the increased total activity of these acid hydrolases (Tappel, 1962) in dystrophic muscle. Phagocytic cells entering regions of lipid peroxidation in tissue would probably be damaged and undergo rupture and further contribute to the release of catabolic enzymes. Overall effects of such events would appear histologically as necrosis and degeneration, and biochemically as

increased turnover, catabolism, and urinary excretion of tissue constituents. Tappel's results showing involvement of lysosomal enzymes in muscular dystrophy constitute the first instance in vitamin E research where its chemical function has been related to the gross pathology which occurs in the deficiency state.

However, in this work, the abnormal vitamin E concentration found in DMD plasma is not significantly correlated with the increased levels of plasma lipid peroxidation products. These results therefore tend to suggest that, although the evidence for a reduction in vitamin E concentration in DMD compared with normal control plasma is strong, it cannot be assumed that low vitamin E is the only, or even the main, cause of increased lipid peroxidation products in DMD blood plasma, whatever their source. It is of interest that no significant difference was observed between vitamin E concentration in adults and children (Table 21 and Fig. 56). The reason for this finding is likely to be that the samples were not from infants (the minimum age for the child group was 9 years) who are claimed to have a lower vitamin E concentration than normal adults (Bieri et al.,1983). It has also been established for several years that RBC of infants show greatly increased susceptibility to haemolysis with H_2O_2 compared to RBC of adults (Rose and Gyorgy, 1951). The increased haemolysis can be reduced to normal adult levels by giving supplements of vitamin E (Gordon and de Metry, 1952).

CAERULOPLASMIN AND TRANSFERRIN IN PLASMA

Slater (1979) outlined some of the critical antioxidant protective mechanisms which are operative at the cellular and subcellular level and these have also been reviewed extensively in the Introduction. If one looks at organisms as a whole, in particular at humans, there are other tiers of protection. Human blood serum has been shown to be an exceedingly powerful antioxidant (Barber, 1961; Vidlakova *et al*, 1972), and it was soon established that this property is unrelated to its vitamin E content. Dormandy and coworkers have shown that serum antioxidant activity is the function of two well defined plasma proteins. One is the iron-free fraction of transferrin; the other, the blue copper protein, caeruloplasmin (Stocks *et al*.,1974a,b). These two proteins were immunoassayed in this work. In DMD plasma where caeruloplasmin levels were raised (Figs. 58-59, Table 23, $P<0.001$) there was also a significant correlation between DMD plasma caeruloplasmin and DMD plasma AOA ($P<0.05$; Table 23). A significant correlation between DMD caeruloplasmin and MDA were also found which confirms the correlation between AOA and MDA (Table 24).

The highly significant correlation between DMD plasma caeruloplasmin and plasma AOA (Table 24) supports the suggestion that this cuproprotein is the dominant variable governing plasma AOA (Gutteridge and Stocks, 1981). However, it is perhaps surprising that no significant correlation was found between

caeruloplasmin and AOA in normal plasma.

Transferrin, on the other hand, was unchanged from normal in DMD plasma (Figs. 62-63) and did not significantly correlate with plasma AOA (Table 28). However, since the AOA of transferrin is a function of its ability to bind iron, it might be expected that available iron binding capacity would be more representative of AOA than its absolute concentration. Unfortunately the available iron binding capacity was not measured in this study. It is possible that these marked increases in DMD may be a consequence of muscle damage and necrosis and possibly of the realese of Fe^{2+} from intracellular stores.

It has been suggested that caeruloplasmin may be an antioxidant enzyme, because caeruloplasmin protects tissue homogenates (Al-Timimi and Dormandy, 1977), phospholipid membranes (Gutteridge, 1977), ox-brain phospholipid liposomes (Gutteridge et al.,1980), DNA (Gutteridge, 1978), deoxyribose (Gutteridge, 1982) and RBC membranes (Lovstad, 1981) from lipid peroxidation damage induced by inorganic iron or by ascorbic acid.

However the mechanism of the antioxidant effect of caeruloplasmin has not been clarified in complex systems such as are found in vivo, and its antioxidant effect may depend on ferroxidase activity (Osaki et al.,1966), ascorbate oxidase activity (Osaki et al.,1964) and O_2^{-o} scavenging activity

(Goldstein et al.,1979). More recently, caeruloplasmin has also been found to protect lipids and RBC membranes from copper-stimulated lipid peroxidation damage (Gutteridge et al.,1980; Lovstad, 1982), supporting an antioxidant function other than that of its ferroxidase activity. Gutteridge and Stocks (1981) have pointed out that ferroxidase activity may not be the major biological role of caeruloplasmin but, nevertheless, an inescapable one. In this respect an analogy can be made with the multifunctional protein haemoglobin. The role of haemoglobin is dioxygen transport, whilst that of caeruloplasmin is iron mobilization, and possibly copper donation. Haemoglobin has enzymic peroxidase activity and caeruloplasmin oxidase activity - both are useful markers for their detection but without apparent biological function (Gutteridge and Stocks, 1981). Finally, haemoglobin acts as an important intracellular blood buffer regulating hydroxyl and hydrogen ion levels. Caeruloplasmin may be said to act as an extracellular "antioxidant buffer" against formation of damaging hydroxyl radicals.

No significant difference was found in this study between caeruloplasmin concentration in adults (75.37±13.49 I.U./litre) and children (79.12±11.90 I.U./litre) (Table 23 and Figs. 58-59), consistent with the finding that the level of caeruloplasmin in children (47.99-95.99 I.U./litre) is in the adult (non-pregnant) range (63.99-143.99 I.U./litre) (Eastham, 1978). In addition, birth caeruloplasmin levels, when measured by immunochemical techniques, are approximately the same as found in adults (Mancini et al.,1956; Natelson, 1980). However, the

concentration (in infants) is much lower when measured by oxidase activity, a mean value of 21.29 I.U./l being reported (Scheinberg et al.,1958). By the age of two years caeruloplasmin levels rise to some what above normal adult levels (approx. 139.17 I.U/litre) and then drop gradually for several years thereafter until they reach adult levels , depending upon the diet. Normal plasma also contained some apocaeruloplasmin (approx. 10.80 I.U/litre) which can be measured by radioimmunoassay (RIA) (Matsuda et al.,1974).

AN OVERVIEW

Investigators into DMD have been hampered by the difficulty of obtaining a uniform sample of skeletal muscle plasma membrane because it constitutes only a very small portion of the membranes of the muscle cell and the "muscle" biopsies themselve tend to be heterogeneous, due to the infiltration of fat and connective tissue.

In this study, CSFs in culture were used on the assumption that they would share the defective gene. Unfortunately, the results obtained from this study of CSFs are incomplete due to the small number of CSF lines available for experimentation. In the discussion that follows I shall integrate and summarize these findings in order to support the idea that lipid peroxidation may be involved in DMD, and to discuss the question of whether the changes observed in DMD CSFs are cause or effect of muscle damage. I shall also indicate the directions in which I consider further investigation could be most usefully made.

In the DMD CSFs, as found for other tissues, such as RBC and muscle, increased lipid peroxidation was demonstrable. The presence of some MDA in CSFs is not unexpected since, like other tissues, some lipid peroxidation occurs as a consequence of normal oxidative metabolism. What is perhaps unusual is that MDA is only measurable in confluent cultures of CSFs sometime after confluency is achieved. However the reason for this finding may be closely related with the cells' aging process.

This study did show that the two DMD CSF lines

consistently produced greater amounts of MDA with time under different experimental condition viz. in washed and unwashed cultures and in cultures challenged with arachidonic acid. Using the MDA assay technique described, MDA was usually undetectable in any of the lines at zero time with the exception of washed DMD CSFs incubated with 20:4 (Fig 24). These findings constitute important evidence supporting the notion that DMD cells are more prone to lipid peroxidation than normal controls, although this may only be apparent when they are under stress of some kind. These findings for whole CSF cultures were not confirmed on examination of the isolated washed particulate fraction (Fig 25-26 and Table 9) although significantly increased lipid peroxidation was evident in whole CSF homogenates from DMD CSFs (Fig 27-32 and Table 10) which suggest that particulate lipid peroxidation may not be entirely a membrane-dependent process but also involves cytoplasmic components.

It was surprising in the light of these findings to discover a higher activity of GSH-dependent antioxidant protein(s) in CSF cytosol from DMD lines compared with normal controls (Fig 39-44 and Table 12). This may be an adaptive response by DMD CSFs to an increased peroxidation challenge. As was suggested for the increased CAT and GSHR activity seen in DMD muscle (Kar and Pearson, 1979).

Several other abnormalities have also been described in CSFs from patients with DMD but they all remain as yet unsubstantiated by other laboratories. These include reports of changes in adhesive properties (Jones and Witkowski, 1979,1981,1983), of alterations in iodination of cell surface

proteins (Burghes et al.,1982), of changes in the biosynthesis of collagen and non-collagen proteins, both in whole CSFs (Ionasescu et al.,1977; Ionasescu and Ionasescu, 1982) and using a cell-free polysome system (Boule et al.,1979), and of structural lesions in lysosomes (Gelman et al.,1981). The cell surface abnormality resulting in affected intracellular adhesiveness could be related to increased lipid peroxidation. Recently, Jacob et al (1980) reported that the abnormalities of aggregation and adhesiveness found in leucocytes appear to be mediated by oxygen free radicals and increased TBA-reactive materials.

If these results for CSFs are substantiated by future work using many more CSF lines this would, indeed, provide strong evidence to suggest that increased susceptibility to lipid peroxidation might be closely linked the primary defect in DMD as well as providing further support for the notion of a generalized membrane defect in the disease. The presence of identical membranous whorls, mitochondrials remnants, and inclusion bodies ultrastructurally in progressive neuromuscular disease (Guggenheim et al.,1982) and in vitamin E-deficient animals suggest that the pathogenesis of the muscle damage is related primarily to lipid peroxidation of muscle membranes (West, 1963; Hawes et al.,1964; Hadlow, 1973) and supports the above hypothesis.

The important findings of the studies on DMD plasma are: (i) increased lipid peroxidation products (MDA,CD and FP) (Fig 45-47 and Table 13); (ii) decreased vitamin E (Fig 56 and Table 20); (iii) increased caeruloplasmin (Fig 58-59). A possible interpretation of these findings is that reduced vitamin E and/or

other factors lead to increased muscle lipid peroxidation. The products of this peroxidation damage (MDA, lipoperoxides themselves?) then escape into the blood stream where their presence or that of Fe^{2+} from damaged muscle tissue induces the synthesis of caeruloplasmin. It is certainly possible that the presence of lipid peroxidation products in the circulation might cause damage to other tissues and perhaps give rise to other "membrane defects". The suggestion was already been made that a circulating factor from DMD patients may be responsible for some RBC changes (Lloyd and Emery, 1981). Could this factor be lipid peroxidation products?

An alternative and equally plausible explanation of the plasma findings might be that an initial event, unrelated to lipid peroxidation, causes membrane damage and allows influx of Ca^{2+} which sets in train the sequence of damaging reactions which lead to muscle necrosis. Perhaps as a consequence of tissue damage (see Halliwell and Gutteridge, 1984) lysosomal and extra-lysosomal enzymes bring about decompartmentalisation of Fe (release from Fe containing proteins by proteases) and that this then acts as the trigger for lipid peroxidation. In other words, the possibility still exists that lipid peroxidation, although a factor in the pathogenesis of DMD may simply be a secondary consequence of muscle damage. Our study nevertheless provides important additional evidence in support of the theory suggesting involvement of lipid peroxidation via active oxygen radical species in the pathogenesis of muscular dystrophies (Omaye and Tappel, 1974; Chau et al.,1975; Francis et al.,1976; Pathode et al.,1976; Kar and Pearson, 1979; Mechler et al.,1984) and

particularly in DMD (Burri et al.,1980; Hunter et al.,1981; Matkovics et al.,1982; Hunter and Amin, 1983; Amin and Hunter, 1984).

Inevitably the results presented in this thesis pose as many questions as they answer especially in the case of CSFs where very limited numbers of CSFs lines were used. In the following final section, I outline the kinds of experiment which might further elucidate the precise role of lipid peroxidation in the degradation and damage of skeletal muscle in DMD.

Future investigations

It is obvious in this work that MDA is detectable in confluent CSF cultures. However the concentration is very small and the assay technique described here was generally not sensitive enough to detect the endogenous levels of MDA present in cultures at the beginning of the incubations. It would clearly be of value to established whether the basal concentration of MDA in unstressed CSFs is higher in DMD lines. A more sensitive technique is needed to evaluate whether such a small change actually occurs or not. Such an approach is now possible using the fluorometric technique (to measure FP) which has been found to be considerably more sensitive than TBA assay. More importantly FP is the most stable peroxidation product.

It would also be of interest to determine whether the endogenous concentration of vitamin E is decreased in DMD CSFs and whether the difference in lipid peroxidation susceptibility could be abolished by supply of suitable amounts of

antioxidants. Such studies with antioxidants might pave the way for therapeutic trials of such compounds in DMD.

That a GSH-dependent antioxidant protein is significantly increased in DMD CSFs is an interesting finding. The identity of this protein was not further investigated in this work but it is unlikely to be any of the antioxidant enzymes investigated by Hunter and Amin (1984) since there were found to be normal. It would be fruitful to determine whether this protein is GSH-S-transferase as suggested by Burk et al (1980) or GSHPx (Christophersen, 1969) or distinct from these two enzymes (Ursini et al.,1982; Mariorino et al.,1982). If the increased peroxidation potential of CSFs is confirmed it would be important to attempt to demonstrate the same phenomenon in cultured muscle cells.

Regarding the plasma studies of lipid peroxidation products, to interpret the probable significance of these results, it will clearly be necessary to establish their origin more precisely and to relate different patterns of peroxidation products (CD,MDA and FP) to the associated or underlying pathological process in DMD muscle or may be other sites. Obviously the situation is complicated and merits a more detailed investigation, especially one which directly involves the use of both DMD and normal muscle which is extremely difficult to obtain. Biopsies are not taken very frequently. They are unpleasant for already distressed patients and the physicians therefore often do not feel that they can take them very often, without extremely good justification. Moreover, it is also difficult to obtain muscle biopsies for normal controls. Thus if

the plasma and CSF's continue to be useful, it will be very helpful to the whole field of investigation.

Furthermore the studies of vitamin E, caeruloplasmin and transferrin are incomplete without measurement together with plasma lipids, ferroxidase activity and iron binding capacity respectively, which remain for future elucidation.

The possibility that damaging, free Fe^{2+} could be released from damaged muscle as well as from the plasma itself (as a result of protein damage in DMD plasma?) is another important area to study. A vital set of experiments which should be carried out is the monitoring of the plasma parameters presented here in other myopathies, and particularly the other muscular dystrophies. This would go a long way towards answering the question as to whether the changes reported here are simply a consequence of non-specific muscle damage and necrosis or are a specific feature of DMD.

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